The Melatonin Rhythm-generating Enzyme: Molecular Regulation of Serotonin *N*-acetyltransferase in the Pineal Gland

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ABSTRACT

A remarkably constant feature of vertebrate physiology is a daily rhythm of melatonin in the circulation, which serves as the hormonal signal of the daily light/dark cycle: melatonin levels are always elevated at night. The biochemical basis of this hormonal rhythm is one of the enzymes involved in melatonin synthesis in the pineal gland—the melatonin rhythm-generating enzyme—serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AA-NAT, E.C. 2.3.1.87). In all vertebrates, enzyme activity is high at night. This reflects the influences of internal circadian clocks and of light. The dynamics of this enzyme are remarkable. The magnitude of the nocturnal increase in enzyme activity ranges from 7- to 150-fold on a species-to-species basis among vertebrates. In all cases the nocturnal levels of AA-NAT activity decrease very rapidly following exposure to light. A major advance in the study of the molecular basis of these changes was the cloning of cDNA encoding the enzyme. This has resulted in rapid progress in our understanding of the biology and structure of AA-NAT and how it is regulated. Several constant features of this enzyme have become apparent, including structural features, tissue distribution, and a close association of enzyme activity and protein. However, some remarkable differences among species in the molecular mechanisms involved in regulating the enzyme have been discovered. In sheep, AA-NAT mRNA levels show relatively little change over a 24-hour period and changes in AA-NAT activity are primarily regulated at the protein level. In the rat, AA-NAT is also regulated at a protein level; however, in addition, AA-NAT mRNA levels exhibit a 150-fold rhythm, which reflects cyclic AMP-dependent regulation of expression of the AA-NAT gene. In the chicken, cyclic AMP acts primarily at the protein level and a rhythm in AA-NAT mRNA is driven by a noncyclic AMP-dependent mechanism linked to the clock within the

pineal gland. Finally, in the trout, AA-NAT mRNA levels show little change and activity is regulated by light acting directly on the pineal gland. The variety of mechanisms that have evolved among vertebrates to achieve the same goal—a rhythm in melatonin— underlines the important role melatonin plays as the hormonal signal of environmental lighting in vertebrates.

I. Introduction

A highly conserved feature of vertebrate physiology is a daily rhythm in melatonin production, with high values occurring at night (Fig. 1). This pattern is seen in all animals, whether they are active during the day or during the night, and is not linked to activity or lifestyle. Rather the rhythm in circulating melatonin is very closely linked to the environment—specifically to light and darkness. The rhythm in melatonin production is important because it entrains behavioral and physiological rhythms to the environmental lighting cycle and controls major

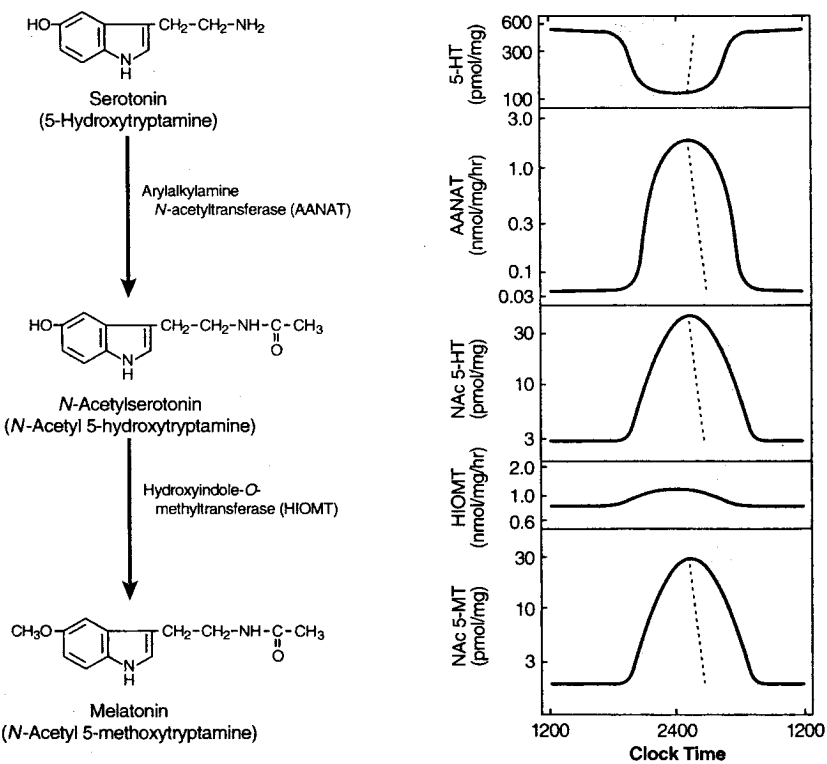

FIG. 1. Rhythms in indole metabolism. Taken from reports in the literature cited in the text, adapted from Klein (1985). The dashed lines represent the effects of an unexpected exposure to light at night.

seasonal changes in vertebrate physiology, including body weight, coat color and quality, and reproductive activity (Arendt, 1995).

Melatonin values remain low during the day with few exceptions, even if animals remain in darkness. Conversely melatonin values are high only during the night, provided animals are in darkness. Unexpected exposure to light at night causes a rapid decrease in melatonin in the pineal gland and in the circulation (Namboodiri *et al.*, 1985). These characteristics typically reflect the action of one or more intrinsic circadian clocks and the influence of light on these clocks. In the case of mammals, rhythms in the pineal gland are known to be regulated by a clock in the suprachiasmatic nuclei (SCN), the master mammalian oscillator, which is connected to the pineal gland by a neural circuit (Klein, 1985). Light acts to adjust the clock and also to block stimulation of the pineal gland. In the chicken several clocks exist, including one in the homolog of the SCN (Cassone and Menaker, 1983; Cassone *et al.*, 1990) and another in the pineal gland (Binkley *et al.*, 1978; Deguchi, 1979a). In more than 20 teleost fish, from fresh or salt water, the pineal organ exhibits clock function (Bolliet *et al.*, 1996). A notable exception is the trout, in which no clock appears to function and pineal melatonin output is turned off by light and turned on by darkness (Gern and Greenhouse, 1988). A homolog of the SCN has not been identified in fish; however, clocks do exist in the retina of some (Falcón and Collin, 1991; Wang and Mangel, 1996).

II. The Melatonin Rhythm-generating Enzyme: Serotonin *N*-acetyltransferase

The rhythm in circulating melatonin is due to a rhythm in melatonin production in the pineal gland (Fig. 1), which is the source of circulating melatonin. Melatonin is synthesized from serotonin, which is present in the pineal gland at a higher concentration than any other tissue, with the possible exception of the Raphe nucleus, the location of serotonergic cell bodies.

The conversion of serotonin to melatonin involves two enzymes, serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AA-NAT, E.C. 2.3.1.87) and hydroxyindole-*O*-methyltransferase (HIOMT, E.C. 2.1.1.4)(Fig. 1). The first *N*-acetylates serotonin to form *N*-acetylserotonin; the second *O*-methylates *N*-acetylserotonin to form melatonin.

The rhythm in melatonin synthesis is generated by a rhythm in the AA-NAT activity (Klein and Weller, 1970). An increase in AA-NAT activity at night decreases pineal serotonin and increases the concentration of *N*-acetylserotonin (Fig. 1). The increase in *N*-acetylserotonin increases the production of melatonin by HIOMT through a mass-action influence; changes in the activity of this enzyme have little influence on the large changes in melatonin production that drive the melatonin rhythm (Klein, 1985). Exposure of the animal to light at night (dashed line in Fig. 1) rapidly decreases AA-NAT activity, with a resulting decrease in

N-acetylserotonin and melatonin and an increase in serotonin (Klein and Weller, 1970; Namboodiri *et al.*, 1985; Klein, 1985).

The close association of changes in AA-NAT activity with changes in circulating melatonin places this enzyme at the critical interface between circadian regulatory mechanisms and melatonin synthesis; because of this, AA-NAT has attracted significant attention. Investigators have been interested in learning more about what causes the large increase in AA-NAT activity at night. Is there an increase only in phosphorylation? Does AA-NAT protein increase? Do changes in AA-NAT activity reflect changes in mRNA encoding the enzyme? Does the mRNA change at all? What is the functional anatomy of the enzyme? These questions went unanswered for 25 years after the rhythm was first described (Klein and Weller, 1970). However, the recent cloning of the enzyme has provided powerful tools to answer some of these questions. This has led to the surprising discovery that a variety of strategies have evolved among vertebrates to accomplish the same goal (i.e., the generation of a nocturnal increase in AA-NAT activity and melatonin production) for the purpose of providing the organism with a reliable hormonal signal of the duration of the night period.

III. Strategies Used to Clone AA-NAT

Two strategies were used to clone AA-NAT. One used an expression method to identify cDNA clones encoding the enzyme (Coon *et al.*, 1995). In this case pools of clones from a night sheep pineal cDNA library were expressed in COS-7 cells and activity was monitored using a highly sensitive assay in which radioactive product was isolated by TLC and detected using a PhosphorImager. After initial identification of a pool of phagemids, the AA-NAT-expressing clone was purified by sib-selection. The substrate specificity of the enzyme encoded by the cDNA clone was found to be essentially identical to that of partially purified ovine AA-NAT (Fig. 2). Notably it had a high capacity to acetylate tryptamine, serotonin, and phenylethylamine and did not acetylate arylamines, including phenetidine. Specific enzyme activity was also recovered when the recombinant isolate was expressed in a bacterial system or in a reticulocyte system, providing evidence that the protein encoded by the selected clone was, in fact, AA-NAT and not a protein that activated a hypothetical AA-NAT pre-existing in COS-7 cells in an inactive form.

The second strategy used to clone AA-NAT was based on the assumption that there is a nocturnal increase in the abundance of mRNA encoding AA-NAT in the pineal gland (Borjigin *et al.*, 1995). cDNA was prepared from day and night pools of pineal mRNA and fragments were generated by PCR using random primers. This was used to prepare a subtracted cDNA library that was enriched with fragments of transcripts preferentially expressed during the night. Clones

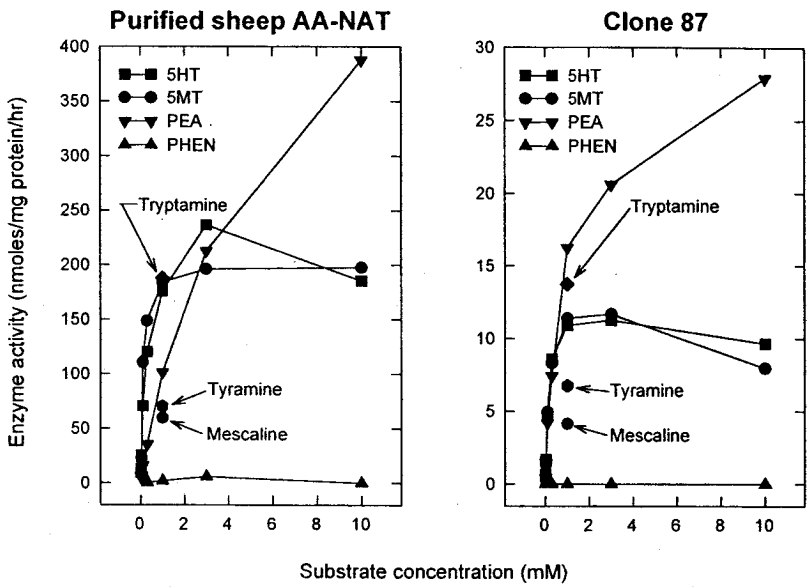

FIG. 2. Substrate specificity of partially purified sheep AA-NAT and cloned ovine AA-NAT (clone 87) expressed in COS-7 cells. Acetylation was determined in 20 µl assays containing the indicated concentration of amine and 40 µM [^{14}C]acetyl CoA (60 Ci/mol). [^{14}C]Acetylated 5-methoxytryptamine (5MT), tryptamine, phenylethylamine (PEA), and phenetidine were extracted into chloroform. [^{14}C]Acetylated mescaline, serotonin, and tyramine were resolved by thin-layer chromatography with acetylated standards. Radioactivity was determined by scintillation counting. The apparent K_m (AcCoA = 40 µM) values for 5-methoxytryptamine with partially purified pineal AA-NAT and with expressed AA-NAT were 90 µM and 120 µM, respectively; those for 5-hydroxytryptamine were 180 µM and 140 µM, respectively. Neither enzyme preparation acetylated 1 mM aniline, sulfamethazine, 2-aminofluorene, isoniazid, *p*-aminobenzoic acid, octopamine, dopamine, norepinephrine (NE), epinephrine, amphetamine, histamine, tryptophan, puromycin, glucosamine, ethanolamine, or chloramphenicol. [Reprinted with permission from Coon, S.L., Roseboom, P.H., Baler, R., Weller, J.L., Namboodiri, M.A.A., Koonin, E.V., and Klein, D.C., *Science* **270,** 1681–1683, 1995. Copyright 1995 American Association for the Advancement of Science.]

were selected from the library based on their ability to identify species of mRNA that exhibited night/day differences in expression in the pineal gland and low levels of expression in other tissues. One of these partial clones was selected and used to identify a full-length clone in a night rat pineal cDNA library; this clone was subsequently found to encode AA-NAT (Borjigin *et al.,* 1995).

The availability of AA-NAT cDNA clones has led to rapid advances in our understanding of the AA-NAT gene, of the encoded mRNA and enzyme protein, and of how expression of the gene is regulated. These are reviewed below.

IV. Characteristics and Evolution of the Gene

A. STRUCTURE OF THE GENE

The portion of the AA-NAT gene encoding the mRNA transcript is approximately 2.5 kb, 1.9 kb, and 3.8 kb in the human, sheep, and chicken, respectively (Fig. 3). The gene is organized into three introns and four exons (Coon *et al.*, 1996b; Klein *et al.*, 1996). The compact nature of the gene is of special interest in planning screening strategies for identification of polymorphisms in humans. This effort has been initiated and methods have been established that allow for complete scanning of the human AA-NAT open reading frame by single-strand conformational polymorphism analysis based on eight PCR reactions. This makes it possible to screen hundreds of samples of human DNA in a routine manner.

B. CHROMOSOMAL LOCALIZATION

The chromosomal localization of the AA-NAT gene has been determined in two species: human and mouse. The mouse AA-NAT gene was located at chromosome 11 in position E1.3–2.3 using fluorescent *in situ* hybridization (Roseboom *et al.*, in preparation). The human AA-NAT gene was located at 17q25 using this and other methods (Coon *et al.*, 1996b). The available data provide no reason to suspect that a second AA-NAT gene is present in either the mouse or human (Roseboom *et al.*, in preparation; Coon *et al.*, 1996b).

A primary purpose in localizing the human AA-NAT gene was to determine

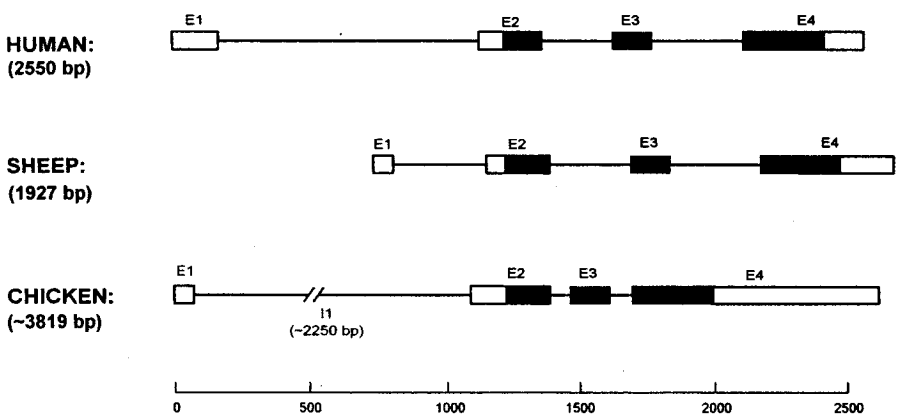

FIG. 3. The AA-NAT gene in three species. Open boxes represent exons, lines represent introns, and solid boxes represent the open reading frame. The accession number of the human gene sequence is U40391. The sequences of the other genes have not been deposited. [After Coon *et al.*, 1996b; from M. Bernard, R. Baler, S.L. Coon, P.H. Roseboom, and D.C. Klein, unpublished results.]

if it was linked to a known inherited and chromosomally mapped disease. This was found not to be the case. If AA-NAT is linked to an inherited disease in humans, it might be as one of several genetic factors influencing a disease state or to a disease for which the chromosomal localization is not yet known (Klein *et al.,* 1997).

Localization of the mouse gene was of interest for two reasons. First, previous studies have concluded that the gene is located on mouse chromosome 11 (Goto *et al.,* 1994). It was of importance to confirm this because the methodology used in these studies to monitor AA-NAT expression could reflect either genetic localization of AA-NAT or the localization of a gene involved in controlling AA-NAT expression. This was of special concern in the mouse because the adolescent/adult pineal gland of some mice, including the C3H/He strain, makes melatonin, whereas that of other strains of mice, including the C57BL/6 mouse, does not (Ebihara *et al.,* 1986,1987; Goto *et al.,* 1989). The pineal gland of the latter is relatively poorly formed, diffuse, and gelatinous (R. Baler and D.C. Klein, unpublished observations).

C. EVOLUTIONARY RELATIONSHIPS

The deduced amino acid sequence of AA-NAT has been determined for species in three phyla: mammals, birds, and fish. From this analysis it is apparent that vertebrate species are closely related (Fig. 4A). Although a high degree of conservation exists within vertebrate forms of AA-NAT, analysis of the protein and DNA sequence databases using either the BLAST or FASTA search programs does not reveal statistically significant similarity to any other characterized protein or DNA sequence in the databases as of November 1996 (Devereux *et al.,* 1984). This is surprising because over 1,000,000 DNA sequences have been entered, including the complete genomes of three species. Such an observation is consistent with the possibility that this enzyme evolved rapidly, late in evolution. For example AA-NAT might have evolved in association with the evolution of melatonin as a signal of darkness in vertebrates. Support for this hypothesis comes from the finding that the enzyme has very high specificity for serotonin and closely related molecules (Fig. 2).

Alternatively, this might not be the case, because AA-NAT is statistically similar to a hypothetical yeast protein (YD8554.04c) identified by DNA sequence analysis (Coon *et al.,* 1995). This sequence has not been expressed and it is not known whether it encodes a functional acetyltransferase. If it were to be found that YD8554.04a encodes a functional arylalkylamine *N*-acetyltransferase, then it is clear that AA-NAT evolved early in evolution.

Although significant sequence similarity is not apparent, extensive sequence analysis has revealed that AA-NAT is weakly related to a growing group of proteins. This relationship is based on similarities limited to two sequences, iden-

A

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                                                pka              ck2     ck2
          1
Human     mstqsthplK PeaprlPpgi pespscQRRH TLPAsEFRCL tPEDAvsaFE
Monkey    mstqsthppK PeaprlPpai s...scQRRH TLPAsEFRCL tPEDAvsaFE
Sheep     mstpsvhclK PsplhlPsgi pgspgrQRRH TLPAnEFRCL tPEDAagvFE
Rat       ..mlsihplK PealhlPlgt seflgcQRRH TLPAsEFRCL tPEDAtsaFE
Chicken   mpvlgavpflK PtplqgPrns p...grQRRH TLPAsEFRCL sPEDAvsvFE
Trout     .......... .......... .......... .......... ..........
Sturgeon  .......... .......... .......... .......... ..........
Consensus ----------K P-----P--- ------QRRH TLPA-EFRCL -PEDA---FE

                                                                 ck2
          51
Human     IEREAFISVl GvCPLyLDEI rHFLTLCPEL SLGWFEEGcL VAFIIGSLWD
Monkey    IEREAFISVl GvCPLyLDEI rHFLTLCPEL SLGWFEEGcL VAFIIGSLWD
Sheep     IEREAFISVs GnCPLnLDEV qHFLTLCPEL SLGWFvEGrL VAFIIGSLWD
Rat       IEREAFISVs GtCPLhLDEI rHFLTLCPEL SLGWFEEGcL VAFIIGSLWD
Chicken   IEREAFISVs GdCPLhLDEI rHFLTLCPEL SLGWFEEGrL VAFIIGSLWD
Trout     .....FVSVs GeCPLtLDEV lnFLsqCPEL SLGWFEEGqL VAFIIGSgWg
Sturgeon  .......... .......... .......... .......... .......LWD
Consensus IEREAFISV- G-CPL-LDE- -HFLTLCPEL SLGWFEEG-L VAFIIGSLWD

                                                pkc
          101
Human     kERLmQEsLT LHrsgGhiaH lHVLAVHRaF RQQGrGpILL WRYLhhLgsq
Monkey    kDRLmQEsLT MHrPgGhiaH lHVLAVHcaF RQQGrGpILL WRYLhhLgsq
Sheep     eERLtQEsLa LHrPrGhsaH lHaLAVHRsF RQQGKGSVLL WRYLhhvgaq
Rat       kERLtQEsLT LHrPgGrtaH lHVLAVHRtF RQQGKGSVLL WRYLhhLgsq
Chicken   qDRLsQaaLT LHnPrGtavH iHVLAVHRtF RQQGKGSILM WRYLqyLrcl
Trout     kERLeQEaMT qHiPetsavH iHVLsVHRha RQQGKGSILL W.........
Sturgeon  qEkLtmDaLT LHkPhGstvH iHVLAVHRtF RQQGKGSILM WRYLqyLrcl
Consensus -ERL-QE-LT LH-P-G---H -HVLAVHR-F RQQGKGSILL WRYL--L---
                                            Motif A
                                                      ck2    pkc
          151
Human     PaVRRAaLMC EdaLVPFYer fsFhavGPCa ItvGsLtFmE LhcslggHpf
Monkey    PaVhRAaLMC EdaLVPFYer fgFhamGPCa ItvGsLsFtE LhcslqgHpf
Sheep     PaVRRAvLMC EdaLVPFYqr fgFhpaGPCa IvvGsLtFtE MhcslrgHaa
Rat       PaVRRAvLMC EnaLVPFYek fgFqamGPCa ItmGsLtFtE LqcslrcHtf
Chicken   PcaRpAvLMC EdfLVPFYek cgFvavGPCq VtvGtLaFtE MqhevrgHaf
Trout     .......... .......... .......... .......... ..........
Sturgeon  PyVRRA.... .......... .......... .......... ..........
Consensus P-VRRA-LMC E--LVPFY-- --F---GPC- ---G-L-F-E -------H--
                                  Motif B
          pka  pkc
          201
Human     LRRNSgc
Monkey    LRRNSgc
Sheep     LRRNSdr
Rat       LRRNSgc
Chicken   MRRNSgc
Trout     .......
Sturgeon  .......
Consensus -RRNS--
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B

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                                                                                                                            motif A                     motif B
                                                                                                                  U..UOU.P.UQ..GUO$.UU        U.UY..UGU....
consensus
AA-NAT/SHEEP        36 FRCLTPEDAAGVFEIEREAF 17 LTLCPELSLGWFV  3 LVAFIIGSLWDEERLTQESLA 1 HRPRGHSAH 0 LHALAVDRSFRQQGKGSVLL 24 VPFYQRFGFHPAG 30
                       :* *  **   ::::*  :*    *  ****  * *:     *:: *:*:    * :* **::          : : *   :* : :    :         :**      :***:*:**    :
YD8554.04c/YEAST    13 IRPLIIEDLKQILNLESQGF 14 LINCPELCSGLFI 12 LIGHIMGTKIPHEYITIESMG 1 LQVES-SNH 2 IHSVVIKPEYQKKNLATLLL 24 IPFYERVGFKIIA 30
PUAC_STRLP          93                                            RMAELSGSRLAAQQQMEGLLA 9 HRPKEPAWF 0 LATVGVSPDHQGKGLGSAVV 23 LPFYERLGFTVTA 19
AAC6_CITDI          78                                                                               LHPLVVRPDYQNKGIGKILL 52 YEFYQKNGYYIVG 22
RIMI_ECOLI          65                                                                               LFNIAVDPDYQRQGLGRALL 26 IALYESLGFNEAT 37
SAT4/CAMCO          98                                                                               IEDIAVCKDFRGQGIGSALI 25 CKFYHNCGFKIGS 23
AAC1_PSEAE         107                                                                               IYDLAVSGEHRRQGIATALI 26 VALYTKLGIREEV 11
YJHQ_ECOLI          81                                                                               LAPLAVIPEYQGMGVGGRLI 20 ATYYPRHGFEPCA 47
ORF5/R46            92                                                                               LHKLAVRRTHAGRGVSSALI 23 RGLYERLGFTHVD 15
ORF/STAAU           35                                                                               LHSIGMLPNYQDKGYGSKLL 26 CHVYEKLGGKNDY 15
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C

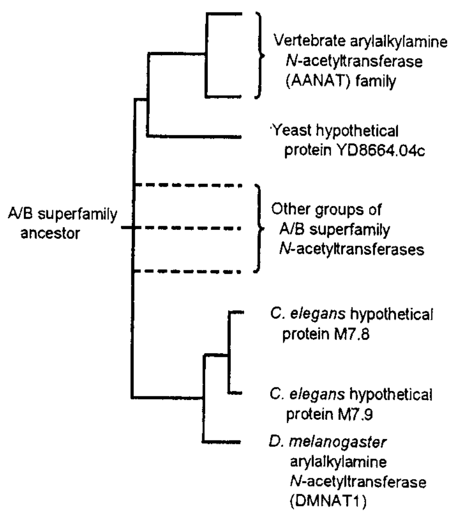


FIG. 4. Amino acid sequence and evolution of AA-NAT. (A) Deduced amino acid sequences of human clone 9 (GenBank accession # U40347), monkey clone MN1, sheep clone 87 (GenBank accession # U29663), rat clone RLL13 (GenBank accession # U38306), chicken clone 9a (GenBank accession # U46502), trout clone T34, and sturgeon clone ST1. The consensus sequence identifies amino acids that are identical in at least five of the available sequences in the species listed. The conserved putative phosphorylation sites are shaded: pKa = cyclic nucleotide-dependent protein kinase; pkc = protein kinase C; ck2 = casein kinase II. Motifs A and B are underlined. (B) Alignment of sheep AA-NAT with sequences of selected known and putative acetyltransferases, as described in Coon *et al.*, 1995. The number of amino acid residues from the protein termini and between the aligned blocks is indicated. Identities between AA-NAT and a yeast hypothetical protein (YD8554.04c) are indicated by asterisks and similarities are indicated by colons. Consensus residues are conserved in the majority of aligned sequences (bold residues conform to the consensus): U = bulky hydrophobic residue (I, L, V, M, F, Y, W); O = small residue (G, A, S); $ = S or T; dot = any residue. (C) A scheme of the relationships between the amino acid sequences of vertebrate arylalkylamine *N*-acetyltransferase (AANAT) and related acetyltransferases. AANAT belongs to a superfamily of known and putative acetyltransferases, in which only two motifs (designated A and B) are universally conserved. There are at least 200 proteins containing these motifs in the current sequence databases, including an arylalklyamine *N*-acetyltransferase found in *D. melanogaster* (DMNAT1). For further details see the text. [A and B reprinted with permission from Coon, S.L., Roseboom, P.H., Baler, R., Weller, J.L., Namboodiri, M.A.A., Koonin, E.V., and Klein, D.C., *Science* **270,** 1681–1683, 1995. Copyright 1995 American Association for the Advancement of Science.]

tified as motif A and motif B (Tercero *et al.,* 1992; Figs. 4B&C). Only a small number of AA-NAT amino acids can be identified as being highly conserved within these motifs. They are assumed to be associated with AcCoA binding because all characterized proteins with these motifs are acetyltransferases and because single-residue substitutions in one of these motifs block catalytic activity. YD8554.04a also contains these motifs (Fig. 4) and this feature is one element that contributes to the statistical similarity to AA-NAT. An interesting member of this family is the *Drosophila* enzyme arylalkylamine *N*-acetyltransferase (DMNAT), which acetylates arylalkylamines (Hinterman *et al.,* 1996); the relative capacity of this enzyme to acetylate arylamines is unknown. The evolutionary relationship of DMNAT to AA-NAT is distant and does not seem to be greater than that to most members of the motif A- and motif B-containing superfamily because similarity is limited to these motifs.

According to these observations AA-NAT belongs to an *N*-acetyltransferase superfamily defined by motif A and motif B (Fig. 4C). It should be noted that this superfamily does not contain all acetyltransferases and at least one other acetyltransferase superfamily exists that contains another identifying motif. A member of this superfamily is arylamine *N*-acetyltransferase (E.C. 2.3.1.5), which is an important drug-acetylating enzyme expressed in the liver and other tissues (Ohsako *et al.,* 1988). It has a high capacity to acetylate arylamines including phenetidine, sulfamethazine, and isoniazid and also acetylates arylalkylamines.

V. The mRNA

A. CHARACTERISTICS

The size of the AA-NAT mRNA transcripts identified to date range from 1.0 to 1.7 kb (Figs. 5–8). Typically a single band is observed in Northern blot analysis. Close examination of high-resolution gels reveals that microheterogeneity exists, such as might be seen if the length of the poly A tail were to change (Roseboom *et al.,* 1996). The most notable exceptions to this single-band pattern are found in some fish, in which multiple bands are clearly observed.

B. TISSUE EXPRESSION

The general pattern of tissue expression seen in all the vertebrates examined is that AA-NAT mRNA is always strongly expressed at night in the pineal gland, as indicated by Northern blot analysis (Figs. 6–8; Coon *et al.,* 1995). In some cases (e.g., the rat), night values are approximately 150-fold greater than day values (Fig. 7). This is not the case, however, in sheep (see below). AA-NAT mRNA appears to be uniformly distributed in the rat pineal gland (Roseboom *et al.,* 1996); in the chicken it is found in follicular cells (modified photoreceptors and parafollicular pinealocytes) but not in interstitial cells, blood vessels, or sym-

pathetic fibers (Fig. 8B). Regulation of AA-NAT mRNA levels is dealt with in detail below in the section on species-specific patterns of regulation.

A second tissue in which significant expression can be detected by Northern blot analysis is the retina (Figs. 5–8). However, night levels of expression vary significantly on a species-to-species basis; ovine and chicken retinal AA-NAT mRNA levels are approximately 25% of those in the pineal, whereas rat retinal levels are 0.5% of those in the pineal. A day/night rhythm in retina AA-NAT mRNA levels is seen in the rat and chicken (Figs. 7 and 8C; Roseboom *et al.,* 1996; Bernard *et al.,* 1997a). Most of the retinal AA-NAT signal is located in the photoreceptor cell bodies in the ovine retina (S.L. Coon and D.C. Klein, unpublished) and the monkey retina (Coon *et al.,* 1996a); labeling in the chicken retina appears to be more generally distributed in the outer nuclear layer, which contains the photoreceptor cells (Fig. 8C; Bernard *et al.,* 1997a). In addition to strong labeling of the photoreceptor cells, weak labeling occurs in the outer region of the inner nuclear layer in monkey and sheep (Coon *et al.,* 1996a; S.L. Coon, S. McCune, and D.C. Klein, unpublished) and in the ganglia cell layer in the chicken retina (Fig. 8C). Retinal AA-NAT is of special interest because in some species, including man, the level of AA-NAT mRNA is about 25% that of pineal levels, whereas the second enzyme in the synthesis of melatonin from serotonin, HIOMT, is nearly undetectable (Coon *et al.,* 1996a; Bernard *et al.,* 1995). This raises the interesting question of the function of retinal AA-NAT. Does it function to regulate retinal serotonin? Does it function to produce trace levels of melatonin? Does *N*-acetylserotonin have a physiological role as a local hormone? Further discussion of retinal AA-NAT is beyond the scope of this chapter; readers are referred to a recent review on the subject (Iuvone, 1996) and to a report describing regulation of retinal AA-NAT mRNA (Bernard *et al.,* 1997a).

In addition to AA-NAT gene expression in the retina and pineal, expression has been detected in the testis (Borjigin *et al.,* 1995), in the pituitary gland (Fig. 6), and in certain brain regions (Figs. 5–7; Coon *et al.,* 1995; Roseboom *et al.,* 1996). It is assumed that this reflects true expression of the AA-NAT gene because rigorous conditions were used to detect the signal, which was the same size as that seen in the pineal gland. In addition AA-NAT activity can be detected in some of these tissues (Coon *et al.,* 1995). Expression of the AA-NAT gene in the brain is of special interest because it raises the possibility that brain serotonin might be metabolized by AA-NAT and that drugs could be developed that could modulate brain serotonin by inhibiting or elevating AA-NAT activity.

VI. The Protein

A. PHYSICAL CHARACTERISTICS

The molecular mass of AA-NAT is ~ 23 kDa, based on the deduced amino acid sequence (Fig. 4); this is consistent with the apparent molecular weight of

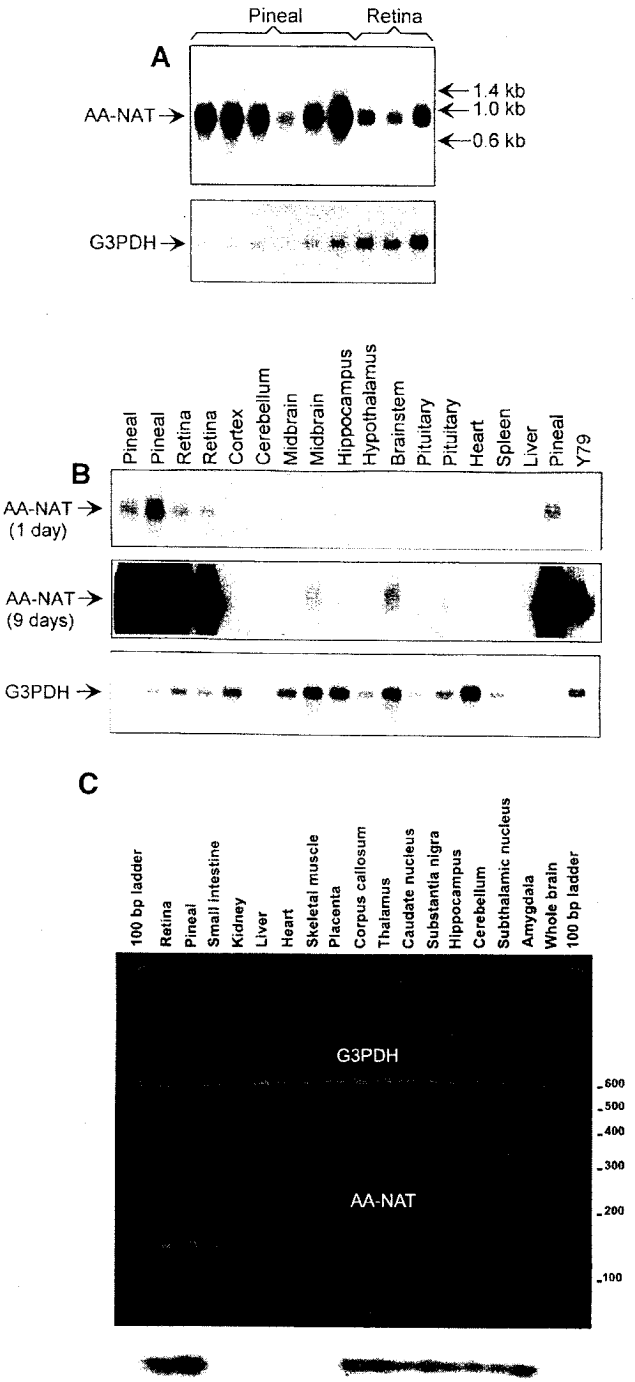

A
Pineal
Retina
AA-NAT
1.4 kb
1.0 kb
0.6 kb
G3PDH
B
Pineal
Pineal
Retina
Retina
Cortex
Cerebellum
Midbrain
Midbrain
Hippocampus
Hypothalamus
Brainstem
Pituitary
Pituitary
Heart
Spleen
Liver
Pineal
Y79
AA-NAT
(1 day)
AA-NAT
(9 days)
G3PDH
C
100 bp ladder
Retina
Pineal
Small intestine
Kidney
Liver
Heart
Skeletal muscle
Placenta
Corpus callosum
Thalamus
Caudate nucleus
Substantia nigra
Hippocampus
Cerebellum
Subthalamic nucleus
Amygdala
Whole brain
100 bp ladder
G3PDH
AA-NAT
600
500
400
300
200
100

FIG. 5. Tissue distribution of human AA-NAT mRNA. (A) Northern blot analysis of various individual pineal gland and retinal samples to demonstrate variability and relative abundance of AA-NAT mRNA. Each lane contains 20 μg of total RNA. The blot was probed with a ^{32}P-labeled, random-primed, full-length AA-NAT cDNA, then stripped and probed for G3PDH. (B) Northern blot analysis of various brain regions and non-neural tissues. Samples of pineal gland, retina, and Y79 cells contain 20 μg of total RNA; all others contain 3 μg of polyA$^+$ RNA. The blot was probed with a ^{32}P-labeled, random-primed, full-length AA-NAT cDNA, and exposed (1 day, top panel; 9 days, middle panel). It was then stripped and probed with G3PDH (1-day exposure). (C) PCR analysis of various brain regions and non-neural tissues. cDNA was amplified using either primers 5192 (CTGTCCCTGGGCTGGTTGGAGGAG) and 5195 (GCGCGGTGCACGGCCAGCACAT) for AA-NAT or primers 2499 (CCACCCATGGCAAATTCCATGGCA) and 2500 (TCTAGACGGCAGGT-CAGGTCCACC) for G3PDH employing 30 or 25 cycles, respectively. Reactions using each set of primers were mixed and products were separated on an agarose gel. The upper image is the ethidium bromide-stained agarose gel of the PCR products. The lower image is an autoradiograph of Southern-blotted PCR products from the same gel probed with a full-length AA-NAT cDNA probe. [Reprinted with permission from Coon, S.L., Mazuruk, K., Bernard, M., Roseboom, P.H., Klein, D.C., and Rodriguez, I.R., *Genomics* **34,** 76–84, 1996. Academic Press, Inc.]

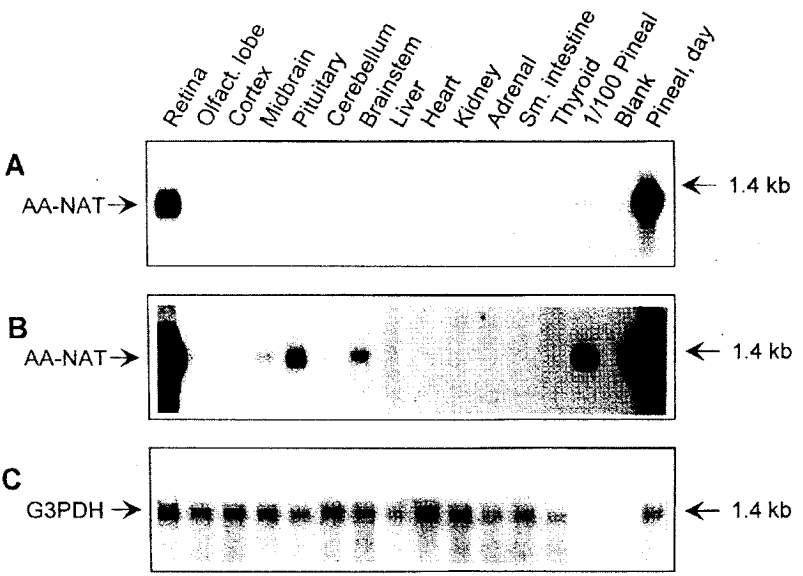
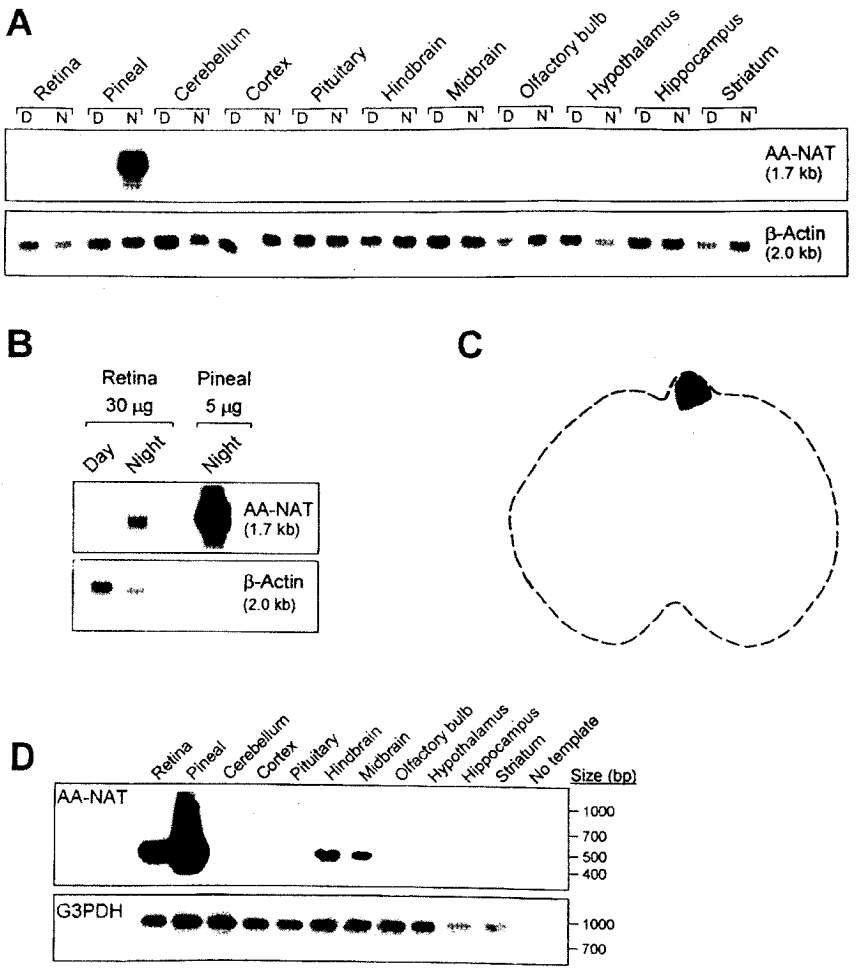


FIG. 6. Tissue distribution of ovine AA-NAT mRNA. Northern blot analysis of various neural and non-neural tissues. Each lane was loaded with 20 μg of total RNA. The blot was probed with a ^{32}P-labeled, random-primed, full-length AA-NAT cDNA, then stripped and probed for G3PDH. Olfact. lobe = Olfactory lobe. (A) A 12-hour autoradiographic image of the blot probed with the insert from clone 87. No other bands of radioactivity were detected. (B) A 96-hour autoradiographic image of the same blot. Faint 3 and 5 kb bands were detected inconsistently in pineal glands and retinae (not shown). (C) A 12-hour PhosphorImager exposure of the blot probed with G3PDH. [Reprinted with permission from Coon, S.L., Roseboom, P.H., Baler, R., Weller, J.L., Namboodiri, M.A.A., Koonin, E.V., and Klein, D.C., *Science* **270,** 1681–1683, 1995. Copyright 1995 American Association for the Advancement of Science.]



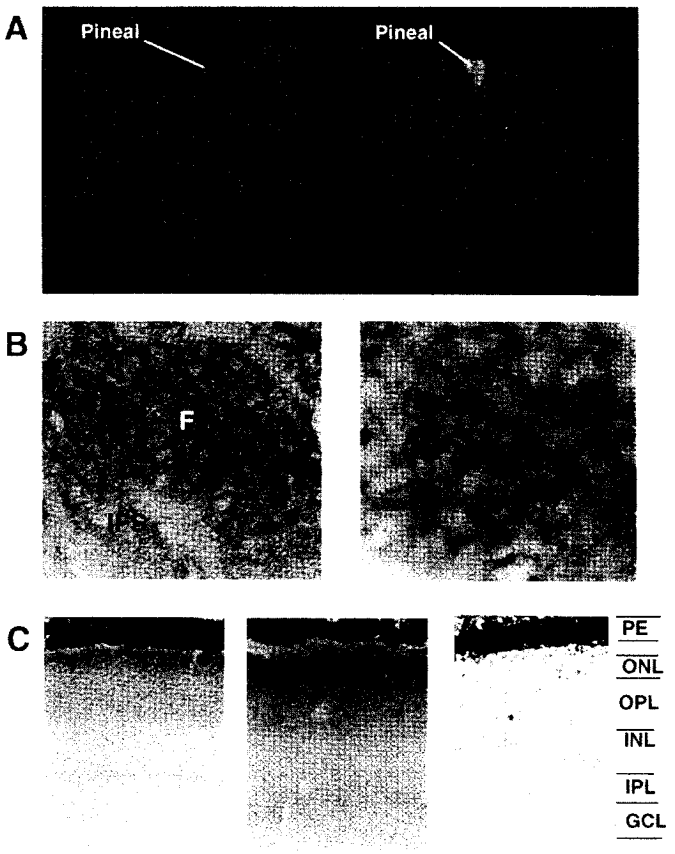
the expressed protein determined from polyacrylamide gel electrophoresis. The calculated pI values of the enzyme in different species ranges from 7.3 to 8.2 (Devereux *et al.*, 1984).

B. CONSERVED FEATURES

The conserved features of the protein can be determined by examination of the full-length (and partial*) deduced amino acid sequences of the available sequences (Fig. 4). The degree of similarity of each form to the human is given in parentheses: monkey (97%), sheep (84%), rat (90%), chicken (76%), sturgeon

FIG. 7. Tissue distribution of rat AA-NAT mRNA. (A) Northern blot analysis performed on RNA obtained from day tissues removed at ~ 1200 hours and night tissues removed at ~ 2400 hours. Rats were housed in controlled lighting LD 14:10 (lights on at 0500 hours). Each lane contains 15 μg of total RNA. Following detection of AA-NAT mRNA, the blot was stripped and probed for β-actin mRNA. (B) Northern blot analysis of day retinal, night retinal, and night pineal AA-NAT mRNA. Each retinal RNA lane contains 30 μg of total RNA obtained from a pool of six retinas and the pineal RNA lane contains 5 μg of total RNA obtained from a pool of five pineal glands. Technical details are described in (A). (C) *In situ* hybridization analysis performed on a coronal section of night brain containing the pineal gland. Tissue was obtained at ~ 2400 hours. AA-NAT mRNA was detected using a pool of three antisense oligonucleotides located in the coding region of clone rLL13. A signal was detected only in the pineal gland. A signal was not detected in similar coronal brain sections prepared from rats killed during the daytime or when sections were probed with sense oligonucleotide controls (data not shown). Results were confirmed with sections from three additional night rat brains. (D) RT-PCR amplification of rat AA-NAT and G3PDH cDNA. cDNAs from different rat tissues obtained at ~ 2400 hours were used as templates for RT-PCR performed with primers for rat AA-NAT (top panel) or G3PDH (bottom panel). The PCR products were first resolved by agarose gel electrophoresis, visualized by ethidium bromide staining, transferred to a Nylon membrane, and hybridized. AA-NAT PCR products were detected with a radiolabeled oligonucleotide located internal to the original PCR primers. G3PDH PCR products were detected with a rat G3PDH probe. Radioactivity was visualized by autoradiography. [Reprinted with permission from Roseboom, P.H., Coon, S.L., Baler, R., McCune, S.K., Weller, J.L., and Klein, D.C., Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. *Endocrinology* **137,** 3033–3044, 1996. © The Endocrine Society.]

FIG. 8. Tissue distribution of chicken AA-NAT mRNA. *In situ* hybridization and Northern blot analysis of AA-NAT mRNA expression in the brain, pineal gland, and retina. (A) *In situ* hybridization analysis of AA-NAT mRNA on sagittal sections of chicken brain containing the pineal gland. Tissues were obtained at ZT 6 (midday; left panel) or ZT 18 (midnight; right panel). AA-NAT mRNA was detected using a [^{33}P]-labeled antisense oligonucleotide located in the coding region of the chicken AA-NAT cDNA clone. A signal was detected only in the pineal gland with this antisense probe but not with the sense probe (data not shown). (B) Cellular distribution of AA-NAT mRNA in the pineal gland. Tissues were obtained at ZT 18. AA-NAT mRNA was detected using a full-length riboprobe labeled with digoxigenin. Bar = 200 μm on the left panel and 100 μm on the right panel. F = follicle; L = lumen; IFS = interfollicular space. (C) Cellular distribution of AA-NAT mRNA in the retina. Tissues were obtained at ZT 6 (left panel) or ZT 18 (center panel). AA-NAT mRNA was detected as described in (B). Right panel: nonspecific binding of the sense digoxigenin-labeled riboprobe in the retina. PE = pigmented epithelium; OS = outer segments; ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL = ganglion cell layer. (D) Northern blot analysis of AA-NAT mRNA. Chickens were housed in LD 12:12 for 2 weeks and then were transferred to constant darkness and sacrificed at CT 18 (subjective night). Northern blot analysis was performed on total RNA (20 μg per lane) obtained from selected brain regions. Following detection of AA-NAT mRNA the blot was stripped and probed for G3PDH. [Reprinted with permission from Bernard, M., Iuvone, P.M., Cassone, V.M., Roseboom, P.H., Coon, S.L., and Klein, D.C. *J. Neurochem.* **68,** 213–224, 1997.]


A
Pineal
Pineal
B
F
C
PE
ONL
OPL
INL
IPL
GCL
D.
Pineal
Retina
Cerebellum
Hyperstriatum
Thalamus
Neostriatum
Medulla
Optic Tectum
Pons
Hypothalamus
AA-NAT
(1.6 kb)
G3PDH
(1.4 kb)

(75%)*, trout (76%)*. Closer examination of the sequences reveals that conservation is not uniform, with the lowest level of conservation apparent at the extreme N-terminal portion of the molecule. The most extensive highly conserved regions are found within a core that extends from the N-terminal PKA site to the end of motif A; within this region there is 81% conservation among all available sequences. Further, there are several short stretches of sequence with 100% conservation in the putative arylalkylamine-binding domain described below (Fig. 9).

1. Protein Kinase A Phosphorylation Sites

Conserved protein kinase A phosphorylation sites are found in the N- and C-terminal regions. These are of potential interest because cyclic AMP is thought to prevent rapid inactivation of the enzyme. It is possible that this involves phosphorylation of these sites and that the rapid, light-induced decrease in AA-NAT activity (dotted line in Fig. 1) reflects the role of phosphorylation in inhibiting proteolysis.

2. Cysteines

There are five conserved cysteines, which are of interest because of the evidence that AA-NAT is inactivated by a mechanism of protein thiol:disulfide exchange. One or more of these thiols could be involved in stabilization/inactivation of the molecule by disulfides and disulfide-containing peptides (Namboodiri *et al.*, 1980,1981). Another possible role of cysteines in AA-NAT is in catalysis. Other acetyltransferases have a cysteine located in the active site (Andres *et al.*, 1988) and one of the three cysteines in the AA-NAT core described above could serve a catalytic role.

3. Histidine-rich Putative Active Site

The finding of a histidine cluster in the center of the molecule is of interest because the imidazole group of histidine has been proposed to act as a catalyst in acetyl transfer from AcCoA to arylalkylamines. This is based in part on the observation that *N*-acetyltryptamine can be generated from tryptamine and AcCoA by the addition of imidazole and that induction of AA-NAT is blocked if 2-fluoro histidine replaces histidine (Klein and Kirk, 1976). Support for the proposal that one of these histidines functions as a catalyst comes from the observation that conversion of His at position 110 to Gln results in inactive protein (Fig. 10). Based on these considerations, this region of the molecule has been designated the putative active site. The local concentration of imidazole within this region would be further increased if the predicted loop is formed around the G(r/k)G sequence in motif A (Schulz, 1992), thereby drawing together the histidines in the flanking regions. In addition to a catalytic role histidines are known to serve a structural function in that they maintain loop configurations in proteins

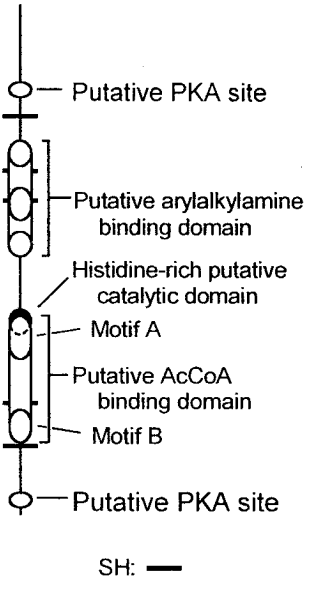


FIG. 9. The conserved features of AA-NAT. The identified features are conserved in all the available deduced amino acid sequences (see Fig. 4). PKA = protein kinase A; SH = cysteine. [Reprinted by permission of the publisher from Klein, D.C., Roseboom, P.H., and Coon, S.L., *Trends Endocrinol. Metab.* **7**, 106–112. Copyright 1996 by Elsevier Science Inc.]

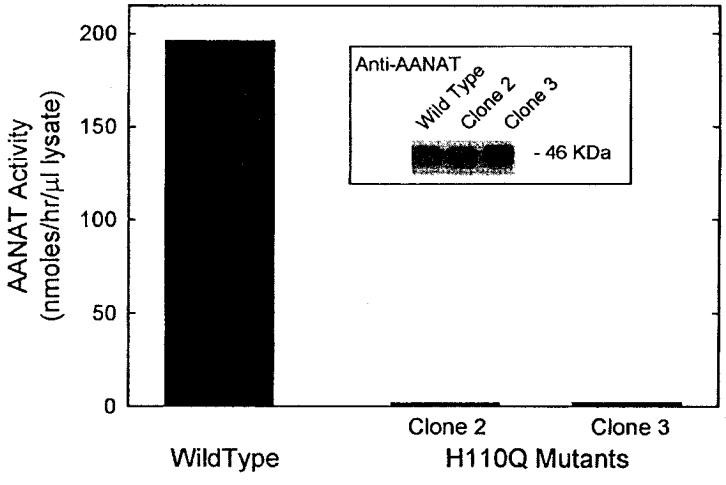


FIG. 10. Evidence that histidine at position 110 is important for AA-NAT activity. A glutathione-S-transferase-AA-NAT fusion protein construct was prepared in the pGEX-4T-1 expression vector (Wild Type) and this was mutated in two identical clones (Clones 2 and 3) to convert the histidine (H) at position 110 to glutamine (Q). Recombinant mutant and wild-type proteins were expressed as glutathione-S-transferase fusion proteins in bacterial cells and AA-NAT enzyme activity was measured in cell lysates. The insert is a Western blot of the cell lysates using anti-AA-NAT antiserum (Ab 2559) to detect immunoreactive protein. This demonstrates that the Wild Type and Clones 2 and 3 expressed AA-NAT protein at similar levels. The bar graphs demonstrate that enzyme with the H-Q mutation was inactive. [From P.H. Roseboom, J. Gastel, and D.C. Klein, unpublished results.]

(Rees *et al.,* 1983). Accordingly some histidine residues in this region could serve a structural function.

4. Putative AcCoA Binding Region

The region that flanks the putative active site on the C-terminus contains motifs A and B. As indicated above these motifs are only found in acetyltransferase molecules. This suggests that they are involved in binding AcCoA, an assumption that is consistent with the predicted loop structure of motif A, characteristic of nucleotide binding sites (Schulz, 1992).

5. Putative Arylalkylamine Binding Site

The region that flanks the active site on the N-terminal side contains three 100%-conserved peptides (11, 17, and 11 AA each) within an ~ 50 AA region. This degree of conservation (78%) across species is consistent with a role for this region in determining substrate specificity and can be considered to be the arylalkylamine binding region. In addition the enzyme's preference for hydrophobic substrates is consistent with the amino acid composition of this region. It should be added that this hydrophobic region might influence binding of AA-NAT to other proteins. For example AA-NAT is known to migrate as a large complex under certain salt conditions (Namboodiri *et al.,* 1986).

VII. Species-specific Characteristics of Regulation

A. SHEEP

Sheep AA-NAT activity exhibits a 7-fold rhythm, which is relatively small compared to the rat. The night/day difference in AA-NAT mRNA is not more than 2-fold (Figs. 6 and 11A); the animal-to-animal variation is such that a statistically significant day/night difference typically is not obvious (S.L. Coon and D.C. Klein, unpublished). Examination of the amount of AA-NAT protein present in the ovine pineal using an antiserum (2345), or with antisera raised against the putative amino terminus phosphorylation region ($AANAT_{21-39}$), indicates that total protein changes about 10-fold over a 24-hour basis, in a pattern essentially identical to that of activity (Fig. 11A). It has also been determined that the rates at which AA-NAT activity and protein disappear following unexpected light at night are essentially identical (Fig. 11A).

These observations indicate that there is a close association between enzyme activity and total protein. Cyclic AMP has been found to regulate ovine AA-NAT activity (Van Camp *et al.,* 1991) through a mechanism that requires *de novo* synthesis of protein but not of mRNA (J. Gastel and D.C. Klein, unpublished). The rapid disappearance of enzyme activity may reflect an inactivation/destabilization step that leads to immediate proteolysis.

The high level of AA-NAT mRNA in the daytime ovine pineal gland may

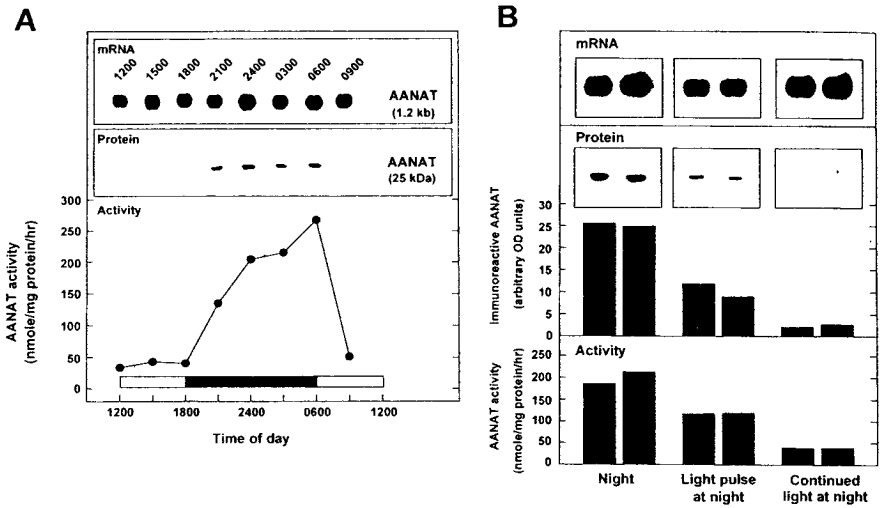


FIG. 11. Relationship of AA-NAT mRNA, enzyme activity, and protein in the sheep. (A) A 24-hour study of AA-NAT mRNA, enzyme activity, and protein in the sheep pineal gland. Sheep were maintained in the field and sacrificed in October. Enzyme activity was measured as described (Parfitt *et al.,* 1976). AA-NAT mRNA was analyzed by Northern blot. Protein was detected by Western blot using antiserum 2345 and quantitated. All three parameters were measured on the same pineal glands. [From J.L. Weller, S.L. Coon, and D.C. Klein, unpublished results.] (B) Effect of light at night on ovine pineal AA-NAT mRNA, enzyme activity, and protein. Sheep were maintained in the field and sacrificed in July. Sheep under constant light condition were kept under artificial lighting during the final night. The other two groups were housed indoors in darkness during the final night. At 0200 some animals were exposed to light, then sacrificed 20–50 minutes later. Animals in the dark and light were sacrificed during this same interval. All three parameters were measured on the same individual pineals. [From J.L. Weller, S.L. Coon, and D.C. Klein, unpublished results.]

explain why sheep melatonin values increase rapidly following exposure to darkness in the night period and remain elevated for relatively long periods of the night (Arendt, 1995). The reason may be that the availability of mRNA at the start of the night period eliminates the lag time required for synthesis of AA-NAT protein to start and, as a result, synthesis of the enzyme starts sooner than if mRNA was not available.

B. HUMAN AND RHESUS MONKEY

Limited analysis of monkey pineal AA-NAT has revealed a large 30-fold night/day difference in enzyme activity but only a 3-fold night/day difference in

AA-NAT mRNA (Fig. 12; Coon *et al.*, 1996a), suggesting that the monkey and sheep may be similar in that regulation does not reflect a primary role of transcription. Analysis of circulating melatonin levels in the Rhesus monkey has revealed that melatonin values increase immediately after lights off, suggesting that enzyme activity increases rapidly and that this reflects the presence of AA-NAT mRNA at the beginning of the night period.

To date, AA-NAT mRNA has been measured in homogenates prepared from six human pineal glands (Fig. 5A). Detectable levels were found in all samples, with a 3-fold difference between minimum and maximum values. It is difficult to draw a firm conclusion from this limited and poorly controlled set of samples as regards rhythmicity. However, we suspect that there might not be a large day/night difference in human AA-NAT mRNA, based on several observations. First, it seems reasonable that if values are at very low levels during the day, as seen in the rat, then a large number of low values would have been seen in the analysis of human pineal glands. Second, many aspects of monkey and human physiology

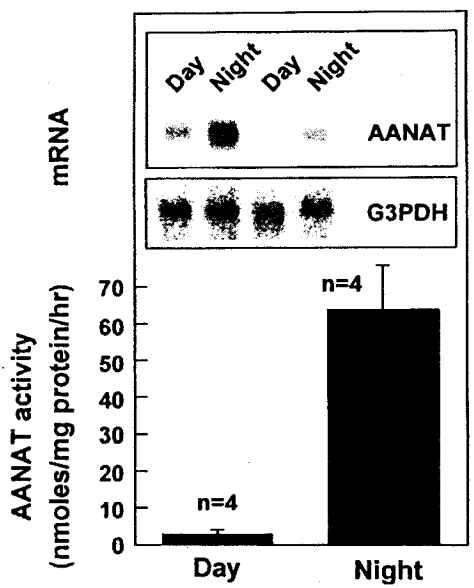


FIG. 12. Rhesus monkey AA-NAT mRNA and activity. Animals were maintained on a 12:12 light:dark cycle. Daytime tissues were obtained between 1100 and 1300 and nighttime tissues between 2300 and 0100. Enzyme activity was measured as described (Parfitt *et al.*, 1976). Northern blot analysis shows mRNA from individual pineal glands. The blot was probed for AA-NAT using a ^{32}P-labeled, random-primed, full-length monkey AA-NAT probe, then stripped and probed for G3PDH. [From S.L. Coon and D.C. Klein, unpublished results.]

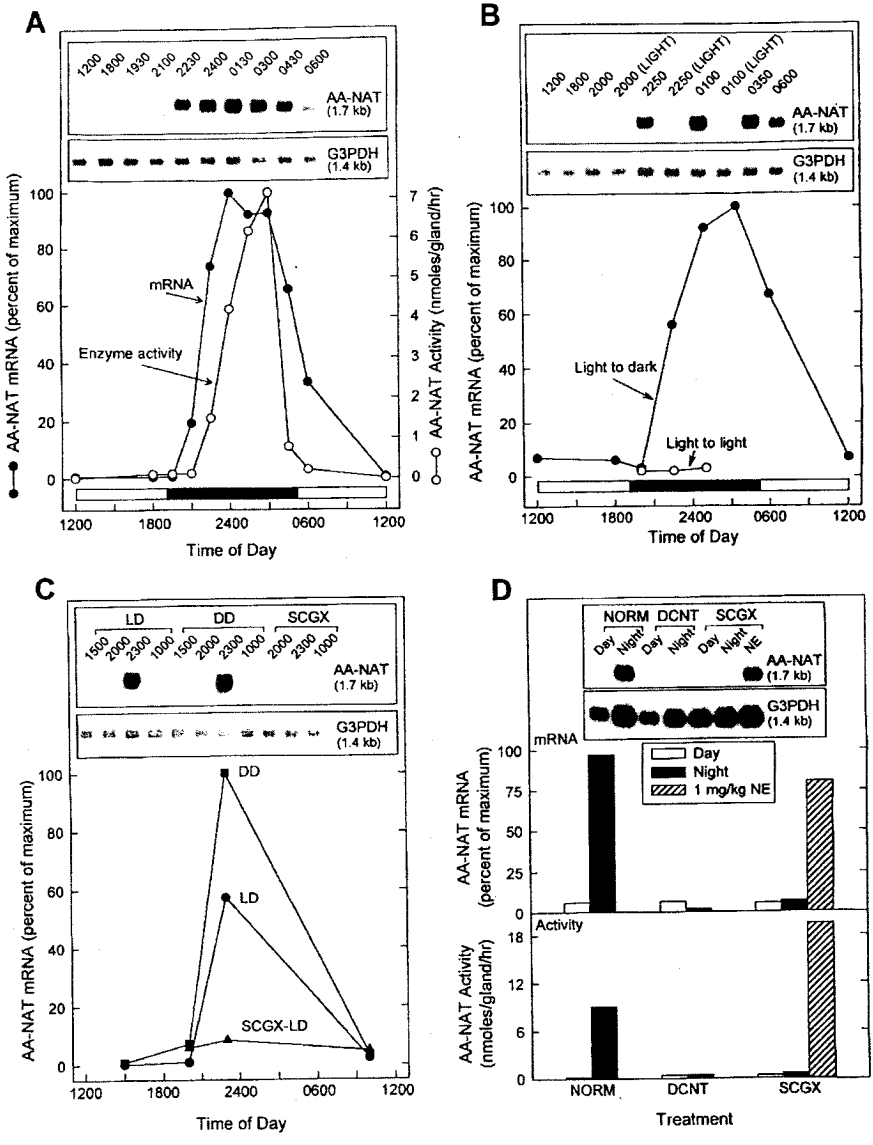
are similar, including the rapid increase in melatonin seen in both following the day → night transition and the rapid decrease seen following exposure to bright light at night (Reppert *et al.*, 1979; Arendt, 1995). Based on this line of thinking it seems reasonable to suspect that human AA-NAT activity is regulated primarily at a posttranscriptional level, as is the case in sheep and in the Rhesus monkey.

C. RAT

The rat pineal is the best-studied vertebrate pineal gland, in part reflecting the popularity of this experimental model, the availability of several highly useful *in vitro* systems, and the large day/night differences in melatonin and *N*-acetyltransferase activity.

Rat pineal AA-NAT mRNA exhibits a large daily rhythm (Figs. 7 and 13). Essentially no signal is detectable during the day and the increase at night is ~ 150-fold. The increase in mRNA is blocked by exposure to light (Fig. 13B), which

FIG. 13. Rat pineal AA-NAT mRNA exhibits a 24-hour rhythm *in vivo* that is under photoneural control. (A) 24-hour rhythm in AA-NAT mRNA and activity. Rats were housed in a controlled lighting environment (LD 14:10) with lights on at 0500 hours. The filled bar indicates when lights were off. The quantitation of mRNA levels is derived from the Northern blot shown in the inset. Each lane contains 5 μg of total RNA obtained from a pool of six pineal glands. The abundance of the transcript was first normalized to G3PDH mRNA and is expressed as a percent of the maximum value (filled circles). AA-NAT enzyme activity was measured in glands taken from rats killed on the same day as those taken for mRNA analysis. The 1200-hour values for AA-NAT mRNA levels and enzyme activity have been plotted twice. Results were confirmed in three independent experiments. (B) Light at night blocks the increase in AA-NAT mRNA. Each lane contains 20 μg of total RNA obtained from a pool of seven pineal glands. Three groups of rats remained in light (open circles) and were sacrificed at the same time as their lights-off counterparts. Normalized mRNA levels are expressed as a percentage of the maximum signal obtained at 0350 hours. The 1200-hour value has been plotted twice. Results were confirmed in three independent experiments. (C) and (D) Daily rhythms in AA-NAT mRNA and enzyme activity are under circadian and neural control. (C) One group of rats was housed in constant darkness (DD) for 3 days, whereas LD and superior cervical ganglionectomized (SCGX) rats were housed in LD 14:10. Each lane contains 20 μg of total RNA obtained from a pool of five to seven glands. Normalized mRNA levels are expressed as a percentage of the maximum signal obtained at 2300 hours in DD animals. (D) Pineal RNA was obtained from normal (NORM), decentralized (DCNT), and SCGX rats sacrificed at ~ 1200 hours (Day) and at ~ 2400 hours (Night). In addition one group of SCGX rats was injected with 1 mg/kg NE (NE) at 2100 hours and sacrificed 3 hours later with the other nighttime experimental groups. Each lane contains 3 μg of total RNA obtained from pools of two pineal glands. mRNA levels are expressed as a percentage of the maximum signal obtained in the Night-NORM sample. Enzyme activity was measured in pineal glands taken from rats killed on the same day as those taken for mRNA analysis. [Reprinted with permission from Roseboom, P.H., Coon, S.L., Baler, R., McCune, S.K., Weller, J.L., and Klein, D.C., Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. *Endocrinology* **137,** 3033–3044, 1996. © The Endocrine Society.]


A
1200 1800 1930 2100 2230 2400 0130 0300 0430 0600
AA-NAT (1.7 kb)
G3PDH (1.4 kb)
AA-NAT mRNA (percent of maximum)
AA-NAT Activity (nmoles/gland/hr)
mRNA
Enzyme activity
1200 1800 2400 0600 1200
Time of Day
B
1200 1800 2000 2000 (LIGHT) 2250 2250 (LIGHT) 0100 0100 (LIGHT) 0350 0600
AA-NAT (1.7 kb)
G3PDH (1.4 kb)
AA-NAT mRNA (percent of maximum)
Light to dark
Light to light
1200 1800 2400 0600 1200
Time of Day
C
LD DD SCGX
1500 2000 2300 1000 1500 2000 2300 1000 2000 2300 1000
AA-NAT (1.7 kb)
G3PDH (1.4 kb)
AA-NAT mRNA (percent of maximum)
DD
LD
SCGX-LD
1200 1800 2400 0600 1200
Time of Day
D
NORM DCNT SCGX
Day Night Day Night Day Night NE
AA-NAT (1.7 kb)
G3PDH (1.4 kb)
mRNA
Day
Night
1 mg/kg NE
AA-NAT mRNA (percent of maximum)
Activity
AA-NAT Activity (nmoles/gland/hr)
NORM DCNT SCGX
Treatment

prevents the transmission of stimulatory signals from the SCN to the pineal gland. The rhythm persists in constant darkness (Fig. 13C), indicating that an endogenous clock drives this rhythm.

The SCN → pineal neural circuit passes through both central and peripheral structures, including the superior cervical ganglia (SCG). Removal of the SCG blocks the nocturnal increase (Fig. 13C), as does decentralization of the SCG (Fig. 13D), which disrupts central innervation of the SCG while leaving the SCG → pineal innervation intact. Together, these observations indicate that the increase in AA-NAT mRNA is regulated by the well-established neural system that controls the pineal gland (Klein, 1985).

Changes in enzyme activity are closely associated with changes in enzyme protein in the rat. Immunoreactive AA-NAT increases at night in parallel to the increase in enzyme activity (Fig. 14A). Similarly exposure to unexpected light at night causes a rapid decrease in enzyme activity and enzyme protein (Fig. 14B); under these conditions there is little change in mRNA (Fig. 14B).

The transmitter regulating AA-NAT mRNA is norepinephrine (NE), as indicated by the observations that NE is contained in pineal nerve endings and is released at night (Klein, 1985). NE treatment of animals that have had their SCG removed results in a large increase in AA-NAT mRNA (Fig. 13D). In addition treatment of pineal glands in organ culture with NE increases AA-NAT mRNA in a dose- and time-dependent manner (Fig. 15). The effects of NE on AA-NAT mRNA are mediated by β-adrenergic → cyclic AMP mechanism, based on the results of *in vitro* studies, which have shown, first, that effects of NE on AA-NAT mRNA are blocked by the β-adrenergic receptor selective antagonist propranolol (Fig. 16A); second, that treatment with the β-adrenergic selective agonist isoproterenol elevates AA-NAT mRNA and activity (Fig. 16B); and third, that treatment with various cyclic AMP protagonists also elevates AA-NAT mRNA (Fig. 16C).

α_1-Adrenergic receptors are known to potentiate β-adrenergic receptor stimulation of AA-NAT activity (Klein *et al.,* 1983) through mechanisms described as "AND" gates in which activation of two systems is required for full effects to be seen (Klein *et al.,* 1992). The molecular basis of these interactions involves elevation of $[Ca^{++}]_i$ and subsequent Ca^{++} activation of protein kinase C (Sugden *et al.,* 1985,1986; Ho *et al.,* 1988). The most striking "AND" gate regulates cyclic AMP production and involves protein kinase C-dependent potentiation of β-adrenergic receptor stimulation of adenylyl cyclase activity (Vanecek *et al.,* 1985; Sugden *et al.,* 1985,1986,1987; Sugden and Klein, 1988). A second interaction involves Ca^{++}-dependent potentiation of the effects of cyclic AMP on AA-NAT activity (Yu *et al.,* 1992). At the present time the roles that α_1-adrenergic receptors, Ca^{++}, and protein kinase C play in the regulation of mRNA and protein encoding AA-NAT have not been established.

The NE-induced increase in AA-NAT mRNA is blocked by actinomycin D

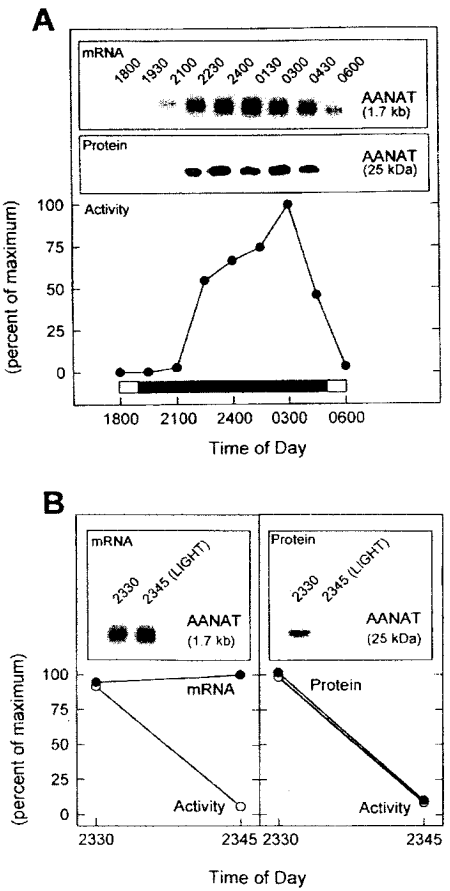


FIG. 14. Relationship of rat AA-NAT enzyme protein and enzyme activity in the rat. Rats were maintained in control lighting conditions as described in Figure 13. (A) Daily rhythm in enzyme activity, immunoreactive protein, and mRNA. The data for mRNA analysis are the same as those shown in Figure 13A. Enzyme activity and immunoreactive protein were measured together in a separate experiment. Rats were sacrificed at the same time points as for the mRNA study, pineal glands were removed, and used for measurement of enzyme activity or analyzed by Western blot using rabbit sera raised against conserved peptides in the putative arylalkylamine binding region (Ab 2559). (B) Effects of light at night. One group of rats was sacrificed at 2330 hours in the dark, a second group of rats was transferred to light and sacrificed 15 minutes later at 2345 hours. Rat pineal glands were removed and processed for enzyme assay and Northern blot analysis (left panel). Each lane contains 10 μg of total RNA obtained from a pool of four pineal glands. In a second study performed under similar conditions (right panel), rats were sacrificed at the same time points. Pineal glands were removed and used for measurement of enzyme activity or immunoreactive protein by Western blot analysis as described in (A). [From J. Gastel, J.L. Weller, P.H. Roseboom, and D.C. Klein, unpublished results.]

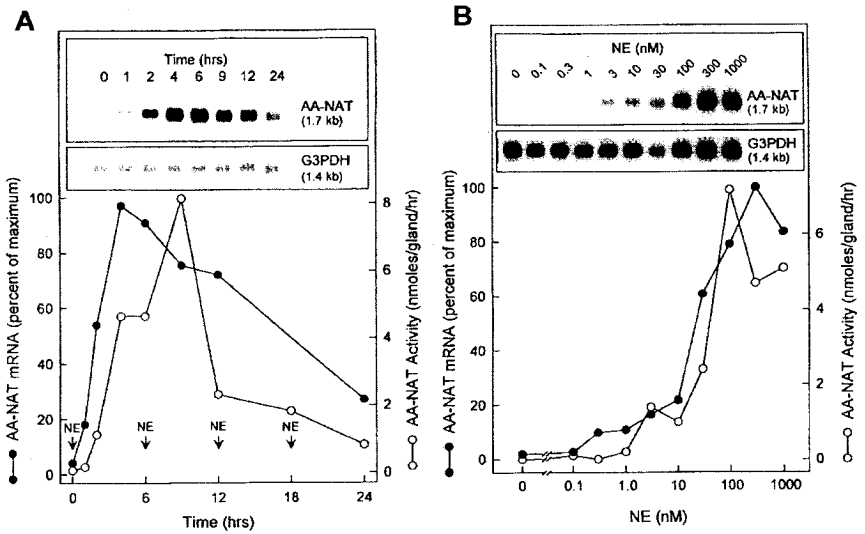


FIG. 15. NE increases rat AA-NAT mRNA in a time- and dose-dependent manner *in vitro*. (A) Time-dependent effects of NE on AA-NAT mRNA. Northern blot analysis of AA-NAT mRNA from pineal glands exposed to NE (1 μM) for the indicated times. Glands were transferred every 6 hours to fresh media containing NE (arrows). Each lane contains 10 μg of total RNA from a pool of six pineal glands. mRNA levels are expressed as a percentage of the maximal stimulation achieved and represent the mean of two independent determinations. AA-NAT activity is expressed as nmoles of acetyltryptamine formed per gland per hour (open circles), with each point representing the mean obtained from three individual glands. (B) Dose-dependent effects of NE on AA-NAT mRNA and activity. Northern blot analysis of AA-NAT mRNA from pineal glands incubated for 6 hours with the indicated concentrations of NE. Each lane contains 3 μg of total RNA obtained from a pool of four pineal glands. AA-NAT mRNA levels are expressed as a percentage of the maximal signal obtained at 300 nM NE (filled circles). [Reprinted with permission from Roseboom, P.H., Coon, S.L., Baler, R., McCune, S.K., Weller, J.L., and Klein, D.C., Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. *Endocrinology* **137,** 3033–3044, 1996. © The Endocrine Society.]

but not by inhibitors of protein synthesis (Fig. 17). This suggests that the key event in turning on expression is not *de novo* synthesis of a transcription factor but an event that does not involve protein synthesis. The most likely event is cyclic AMP-dependent phosphorylation of a transcription factor that binds to the AA-NAT promoter. This hypothesis is supported by several observations. First, NE-treatment causes phosphorylation of the cyclic AMP response element (CRE) binding protein CREB (Fig. 18; Roseboom and Klein, 1995). This response to NE is time dependent (Fig. 19A) and dose dependent (Fig. 19B) and is blocked by the protein kinase A antagonist Rp-8-CPT-cAMPS (Fig. 20).

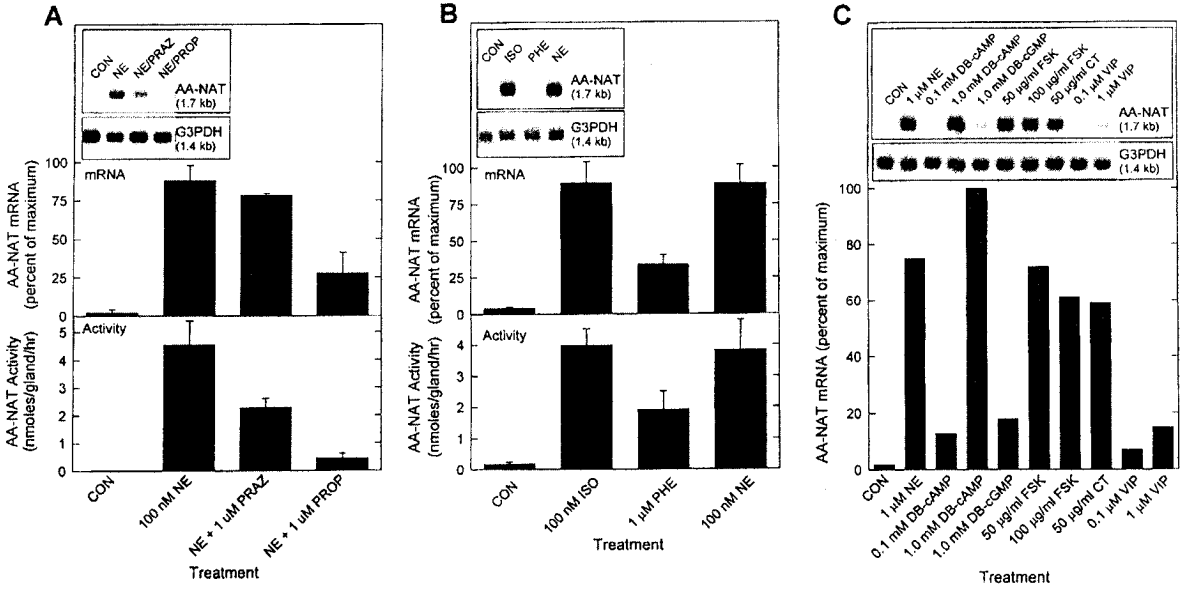


FIG. 16. NE-induced increase in rat AA-NAT mRNA and activity is produced primarily through a β-adrenergic receptor-mediated increase in cAMP. (A) Effects of adrenergic antagonists on NE stimulation of AA-NAT mRNA and activity. Northern blot analysis of AA-NAT mRNA was performed on pineal glands that had been pretreated with 1 μM prazosin (PRAZ) or propranolol (PROP) for 30 minutes prior to 6 hours of stimulation with 100 nM NE. Each lane contains 3 μg of total RNA obtained from a pool of two pineal glands. mRNA levels are expressed as a percentage of the maximal signal obtained with 100 nM NE. Values for mRNA levels and enzyme activity represent the mean of three determinations ± standard error of the mean (SEM). (B) Effects of adrenergic agonists on AA-NAT mRNA and activity. Pineal glands were incubated with the indicated concentration of isoproterenol (ISO), phenylephrine (PE), or NE for 6 hours. Each lane contains 3 μg of total RNA obtained from a pool of two pineal glands. Values for mRNA levels and enzyme activity represent the mean of three to four determinations ± SEM. (C) Effect of cyclic nucleotide protagonists and VIP on AA-NAT mRNA. Pineal glands were cultured for 6 hours with the indicated concentrations of NE, dibutyryl-cAMP (DB-cAMP), dibutyryl-cGMP (DB-cGMP), forskolin (FSK), cholera toxin (CT), and vasoactive intestinal polypeptide (VIP). Each lane contains 10 μg of total RNA obtained from a pool of six pineal glands. [Reprinted with permission from Roseboom, P.H., Coon, S.L., Baler, R., McCune, S.K., Weller, J.L., and Klein, D.C., Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. *Endocrinology* **137,** 3033–3044, 1996. © The Endocrine Society.]

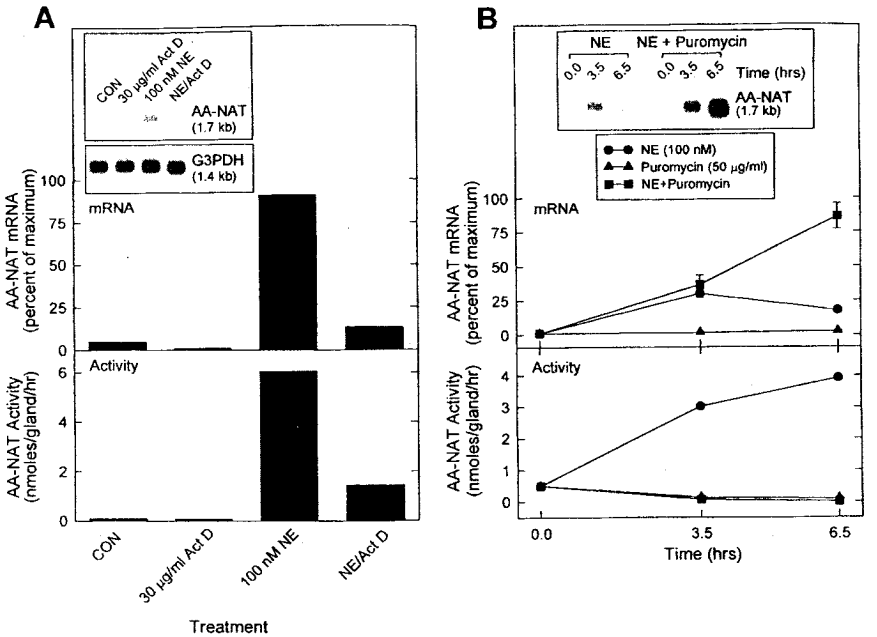


FIG. 17. NE-induced increase in rat AA-NAT mRNA requires gene transcription but not protein synthesis. (A) Actinomycin D blocks the NE-induced increase in AA-NAT mRNA and activity. Glands were pre-incubated with 30 μg/ml actinomycin D (Act D) for 1 hour prior to treatment with 100 nM NE for 6 hours. Each lane contains 3 μg of total RNA obtained from a pool of two pineal glands. Enzyme activity represents the mean of three individual glands. (B) Puromycin does not block the NE-induced increase in AA-NAT mRNA. Northern blot analysis of AA-NAT mRNA from pineal glands exposed to 100 nM NE for the indicated times with or without the simultaneous addition of 50 μg/ml puromycin. Each lane contains 3 μg of total RNA obtained from a pool of two pineal glands. The mRNA levels are expressed as a percentage of the maximal signal obtained with NE + puromycin at 6.5 hours and represent the mean of three determinations ± SEM. AA-NAT activity represents the mean from two individual glands. [Reprinted with permission from Roseboom, P.H., Coon, S.L., Baler, R., McCune, S.K., Weller, J.L., and Klein, D.C., Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. *Endocrinology* **137,** 3033–3044, 1996. © The Endocrine Society.]

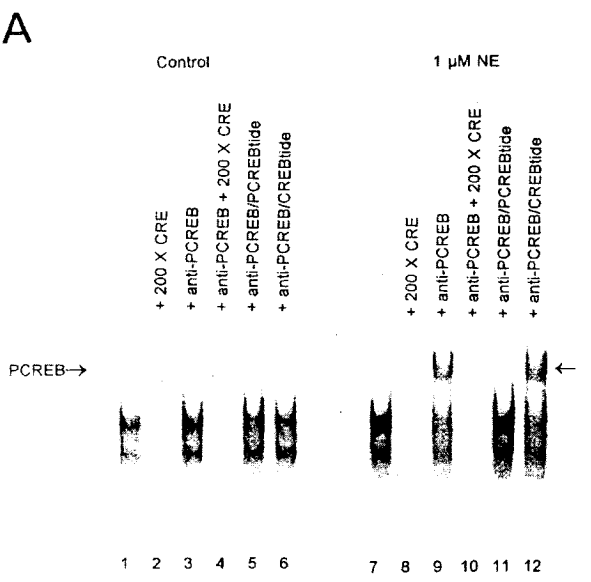
Analysis of the AA-NAT gene has identified a portion of the promoter region that supports cyclic AMP-dependent expression in primary rat pinealocytes (Fig. 21; Baler *et al.*, 1997). This region is of special interest because it contains a CRE, identified as the natCRE, which differs from the canonical CRE sequence by a single base. In addition an inverted CCAAT box is located two helical turns from the natCRE. Both sites appear to mediate cyclic AMP-induced expression of the AA-NAT gene because mutagenesis of either site alone reduces the ability

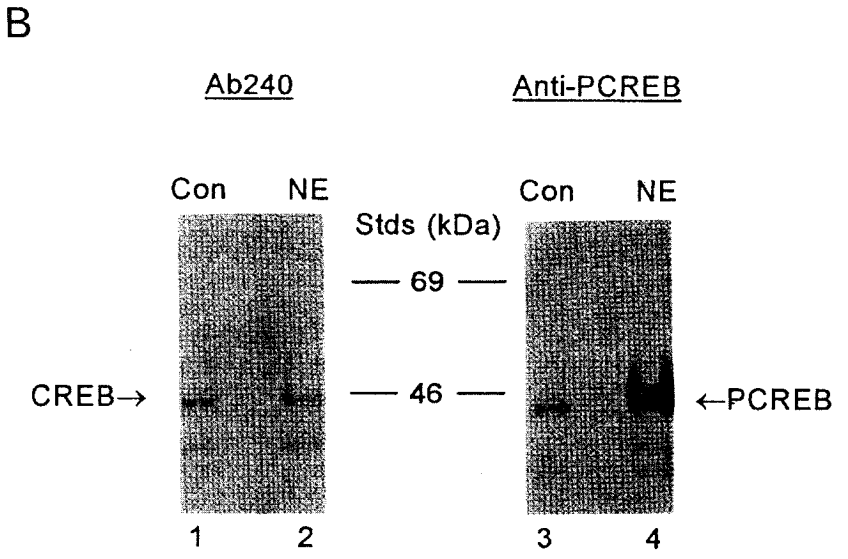
of cyclic AMP to activate expression, whereas the combined mutation results in the loss of detectable cyclic AMP-dependent reporter gene activity (Fig. 22).

The magnitude of the increase in AA-NAT mRNA is increased if induction takes place in the absence of protein synthesis (Fig. 17). This suggests that the increase in mRNA is normally attenuated by the appearance of an inhibitory protein, perhaps a transcriptional repressor. Fos-related antigen 2 (Fra-2) is a likely candidate to play such a role. Fra-2 heterodimerizes with members of the Jun family to form a subtype of AP-1 transcription complexes that binds strongly to AP-1 sites without stimulating transcription (Wisdom and Verma, 1993). The Fra-2 protein is co-expressed with AA-NAT activity at night in the intact animal and in organ culture in response to NE or cyclic AMP (Figs. 23 and 24; Baler and Klein, 1995). More importantly a Fra-2-containing AP-1 DNA binding activity is dramatically increased at the time when AA-NAT mRNA abundance reaches its peak. Thus Fra-2-containing AP-1 complexes could bind to one or several AP-1 sites in the AA-NAT promoter, effectively blocking transcription. During the day the system would be reset for adrenergic stimulation at night because Fra-2 disappears rapidly during the early hours of the day period.

A second negative transcription factor that controls expression of AA-NAT is ICER, the inducible cyclic AMP early repressor (Stehle *et al.,* 1993). ICER and Fra-2 are similar in that mRNA encoding both increase at night, coincident with the increase in AA-NAT mRNA. However, ICER protein exhibits little or no marked day/night rhythm in abundance under typical lighting cycles in which the AA-NAT and Fra-2 rhythms are robust. This argues against a dynamic role

FIG. 18. NE-induced CREB phosphorylation in the rat pineal gland in organ culture. Pineal glands were incubated in control media or media containing 1 μM NE for 30 minutes. (A) Gel mobility-shift assay reactions contained pineal gland extract and ^{32}P-CaCRE probe alone (lanes 1 and 7) or also included 3.5 pmol of consensus CRE oligonucleotide (lanes 2 and 8), anti-PCREB antibody (0.3 μg; lanes 3 and 9), anti-PCREB antibody and 3.5 pmol of consensus CRE oligonucleotide (lanes 4 and 10), anti-PCREB antibody that had been pre-adsorbed with PCREBtide (0.3 μg; lanes 5 and 11), or anti-PCREB antibody that has been pre-adsorbed with CREBtide (0.3 μg; lanes 6 and 12). Antisera (0.15 μg/μl) were pre-adsorbed with peptide (0.3 μg/μl) by incubation for 20 hours at 4°C on an end-over-end rotator. The arrow indicates the location of the supershifted band. The lower half of the gel containing the unbound probe is not included in the image. The supershifted bands were only seen in extracts obtained from NE-treated pineal glands and the appearance was blocked in the presence of excess unlabelled CRE or when the anti-PCREB antibody was pre-adsorbed with the PCREBtide. (B) Immunodetection of NE-induced CREB phosphorylation in pineal nuclear extracts. Pineal nuclear proteins were extracted, resolved by SDS-PAGE, electroblotted, and immunodetected. To detect CREB one half of the blot was probed with Ab240 (1:500); to detect phosphoCREB the other half of the blot was probed with anti-PCREB (0.2 μg/ml). Treatment with 1 μM NE for 30 minutes increased anti-PCREB immunoreactivity in rat pineal nuclear extracts but did not alter the amount of total CREB protein. [Reprinted with permission from Roseboom, P.H., and Klein, D.C., *Molec. Pharmacol.* **47**, 439–449, 1995.]


A
Control
1 μM NE
+ 200 X CRE
+ anti-PCREB
+ anti-PCREB + 200 X CRE
+ anti-PCREB/PCREBtide
+ anti-PCREB/CREBtide
PCREB→
←
1 2 3 4 5 6 7 8 9 10 11 12


B
Ab240
Anti-PCREB
Con
NE
Con
NE
Stds (kDa)
69
46
CREB→
←PCREB
1 2 3 4

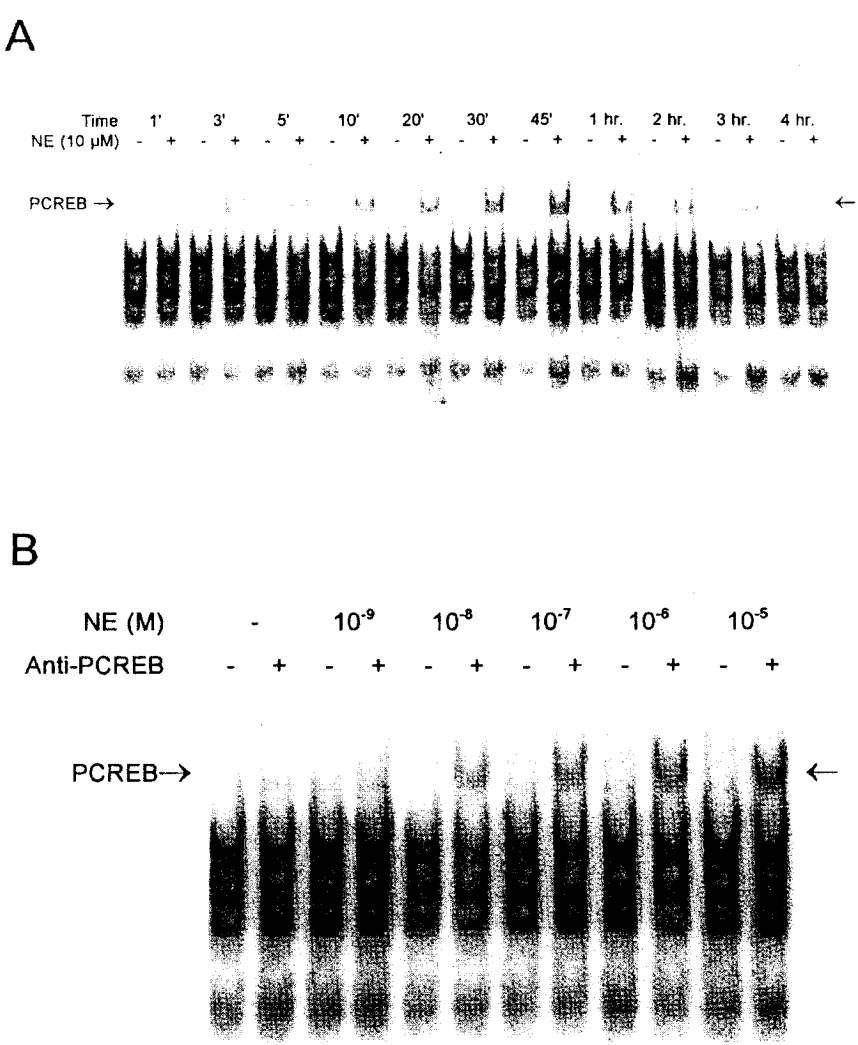


FIG. 19. Characteristics of NE-induced increase in CREB phosphorylation in rat pineal glands in organ culture. (A) Time dependence. Pineal glands were incubated under control conditions and then transferred to control media or media containing 10 μM NE. Pineal glands were incubated for the indicated times and the gel mobility-shift assay was performed with ^{32}P-CaCRE. The supershifted band appeared 1 minute after NE stimulation, reached a maximum after 30–45 minutes, and displayed a decrease after 2 hours. (B) Dose dependence. Pineal glands were incubated under control conditions and then transferred to media containing NE at the indicated concentrations and incubated for 20 minutes. The gel mobility-shift assay was performed with ^{32}P-CaCRE. The supershifted band can be detected in preparations of tissue treated with a concentration of 10 nM NE; the maximal response is obtained at 100 nM. Similar dose-response curves were obtained in four separate studies. [Reprinted with permission from Roseboom, P.H., and Klein, D.C., *Molec. Pharmacol.* **47,** 439–449, 1995.]

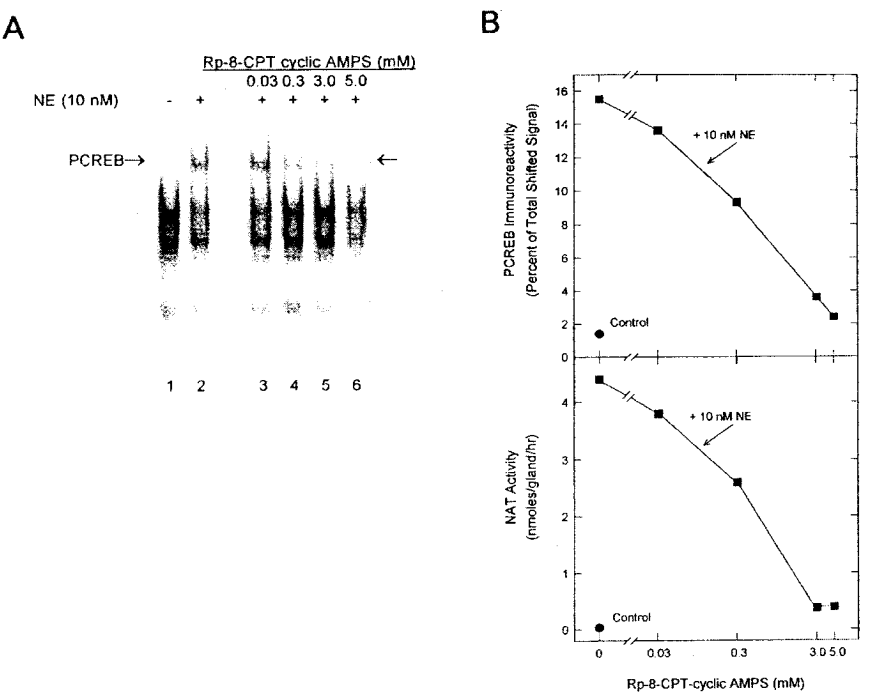


FIG. 20. The PKA antagonist Rp-8-CPT-cyclic AMPS inhibits NE stimulation of rat pineal CREB phosphorylation and of NAT activity. Pineal glands were incubated under control conditions and after 36 hours the glands were transferred to 40 μl of control media or media containing the indicated concentration of Rp-8-CPT-cyclic AMPS in a 96-well plate. The incubation was continued for 18 hours and then glands were transferred to fresh media containing the same concentrations of antagonist in a table-top incubator. After an additional 2-hour incubation, NE was added to the media to a final concentration of 10 nM. The incubation was continued for 30 minutes to measure phospho-CREB and for 4 hours to measure NAT activity. (A) The gel mobility-shift assay was performed with ^{32}P-CaCRE. (B) The concentration effect curves for Rp-8-CPT-cyclic AMPS inhibition of NE-stimulated CREB phosphorylation and NAT activity are compared. The intensity of the phospho-CREB supershifted signal is expressed relative to the intensity of the signal for all shifted bands in (A). This includes the supershifted signal plus the signal for all four shifted bands. NAT activity is expressed as the nmoles of ^{3}H-acetyltryptamine produced per gland per hour. The values for NAT activity represent the average obtained from four glands in a single experiment. Rp-8-CPT-cyclic AMPS inhibited NE stimulation of CREB phosphorylation and NAT activity with a similar potency ($IC_{50} \cong 300\mu M$). [Reprinted with permission from Roseboom, P.H., and Klein, D.C., *Molec. Pharmacol.* **47,** 439–449, 1995.]

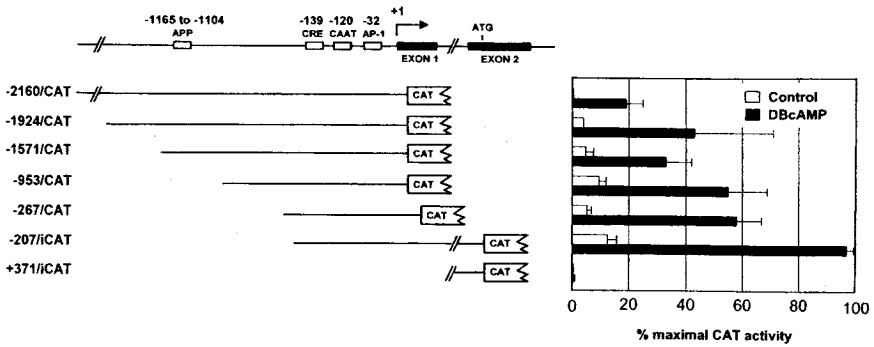


FIG. 21. Functional analysis of the rat AA-NAT promoter. Deletion analysis of the rat AA-NAT promoter. Nested fragments of the AA-NAT promoter were generated by PCR, placed in front of the bacterial choline acetyl transferase (CAT) gene, and transfected into primary pinealocytes using lipofectamine and a defective adenovirus particle to enhance transfection (Kunihiko *et al.*, 1993). Eighteen hours later individual cultures were stimulated with DBcAMP (1 mM) or left untreated. Whole-cell extracts were prepared 48 hours later and CAT enzyme activity was measured. Transfection efficiency was assessed by co-transfection with a Rous sarcoma virus-β-galactosidase construct (RSV-βgal). [Reprinted with permission from Baler, R., Covington, S., and Klein, D.C., *J. Biol. Chem.* 272, 6979–6985, 1997.]

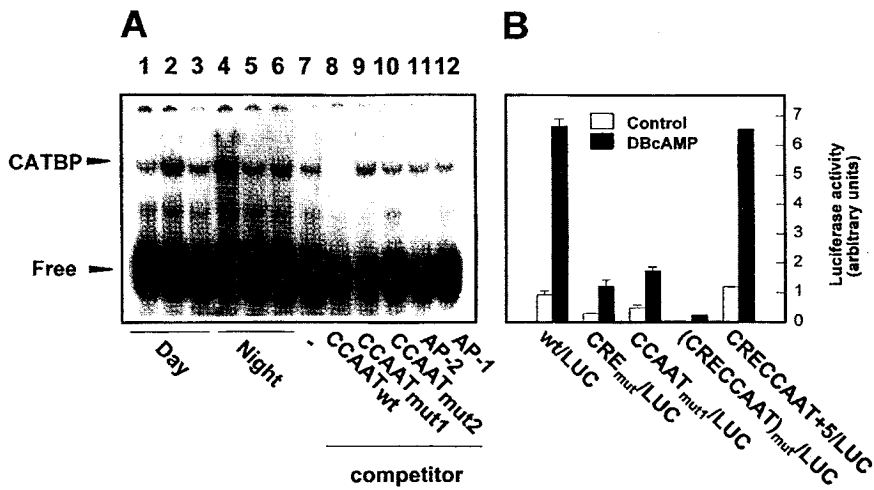


FIG. 22. The inverted CCAAT box in the rat AA-NAT promoter co-participates with natCRE to achieve full cyclic AMP inducibility. Effect of single vs double natCRE/natCCAAT mutagenesis on AA-NAT promoter cAMP responsiveness. The natCCAAT element was mutagenized in the wild-type ($CCAAT_{mut}$/CAT) and in the natCRE mutant ($CRECCAAT_{mut}$/CAT) vectors. An additional construct (CRECCAAT + 5/CAT) contained a 5 bp insertion between natCRE and natCCAAT. The mutated promoters were fully sequenced, spliced in front of the luciferase reporter gene (to yield $CCAAT_{mut}$ /LUC, $(CRECCAAT)_{mut}$ /LUC, and CRECCAAT + 5/LUC), and transfected into primary pinealocytes. Parallel transfections were carried out with wt/LUC and CRE_{mut}/LUC. In selected experiments cultures were co-transfected with a CAT reporter vector driven by the dynorphin promoter construct to provide an internal standard for transfection efficiency. Cultures were stimulated with DBcAMP (1 mM) or left untreated for 48 hours before assessing luciferase and CAT activities. [Reprinted with permission from Baler, R., Covington, S., and Klein, D.C., *J. Biol. Chem.* 272, 6979–6985, 1997.]

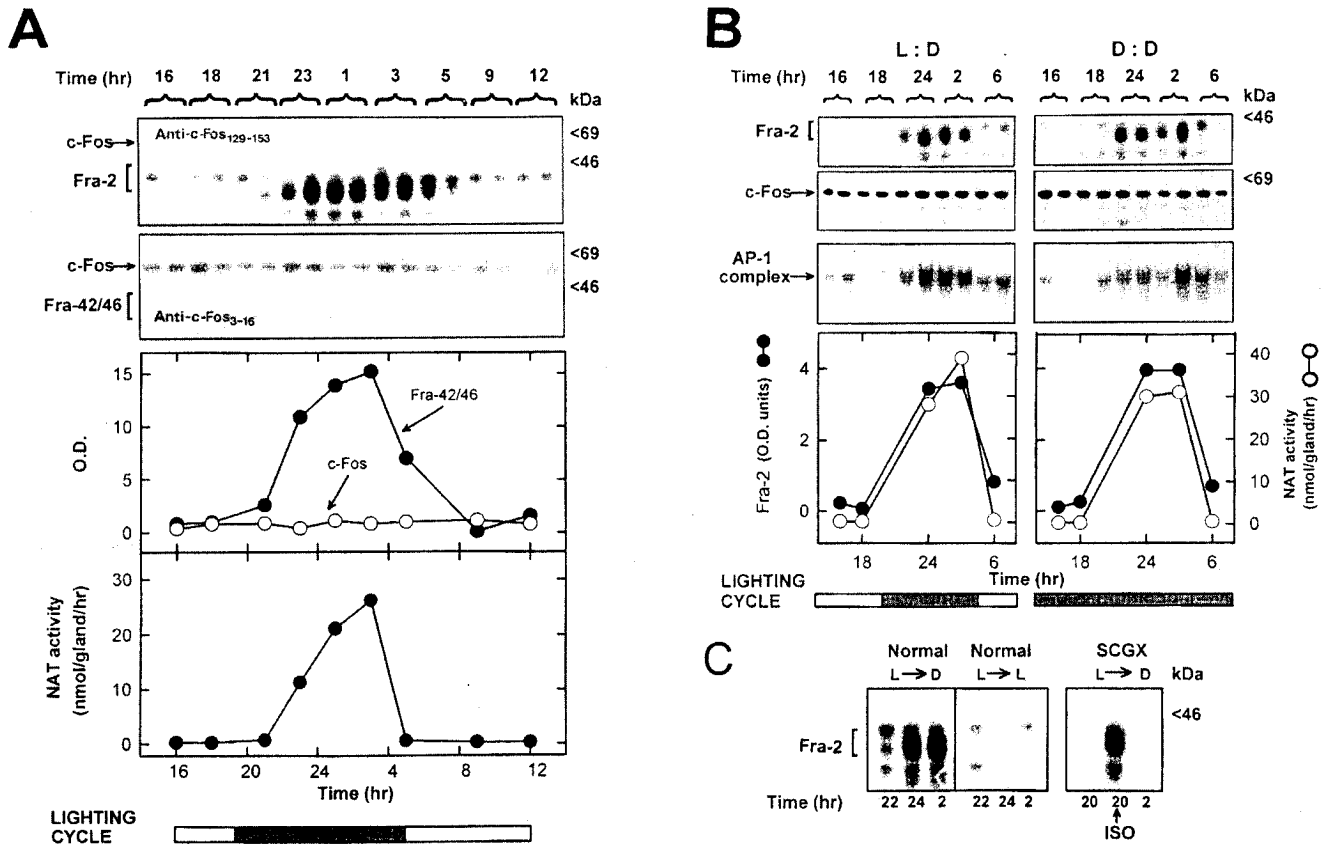

A
Time (hr)
16 18 21 23 1 3 5 9 12
kDa
c-Fos
Anti-c-Fos129-153
Fra-2
<69
<46
c-Fos
<69
Fra-42/46
<46
Anti-c-Fos3-16
O.D.
Fra-42/46
c-Fos
NAT activity (nmol/gland/hr)
16 20 24 4 8 12
Time (hr)
LIGHTING CYCLE
B
L : D
D : D
Time (hr)
16 18 24 2 6
16 18 24 2 6
kDa
Fra-2
<46
c-Fos
<69
AP-1 complex
Fra-2 (O.D. units)
NAT activity (nmol/gland/hr)
18 24 6
18 24 6
Time (hr)
LIGHTING CYCLE
C
Normal L→D
Normal L→L
SCGX L→D
kDa
Fra-2
<46
Time (hr)
22 24 2
22 24 2
20 20 2
ISO

FIG. 23. Circadian rhythm and photoneural regulation of rat pineal Fra-2. Animals were sacrificed at the indicated time of the day. Glands were removed and rapidly frozen on solid CO_2. Horizontal bars in A and B represent lighting (light = open bar; dark = shaded bar). (A) Proteins in whole pineal extracts were subjected to immunoblotting with a rabbit affinity purified anti-pan-Fos serum, anti-c-$Fos_{129-153}$ serum (Quinn *et al.*, 1989; top panel). The second antiserum was horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin. The blot was stripped and re-exposed to a polyclonal affinity-purified c-Fos-specific antiserum, anti-human c-Fos_{3-16} antiserum (SC-52, Santa Cruz Biotechnology, Santa Cruz, CA; second from top panel), and processed as above. Each lane represents a pool of two glands. The third panel is a densitometric quantitation of Western blot data (solid circles = Fra-2; open circles = c-Fos). The fourth panel presents AA-NAT activity in the extracts. (B) Analysis of pineal glands from animals housed in a 14:10 light/dark lighting cycle (L:D) or in constant darkness for 4 days (D:D). Pineal extracts were analyzed (from top to bottom) for anti-c-$Fos_{129-153}$ and anti-c-Fos_{3-16} immunoreactivities; AP-1 DNA binding activity (free probe is not shown), and AA-NAT activity (open circles). (C) Effect of continuous light exposure at night and superior cervical ganglionectomy (SCGX) on Fra-2. Groups of four pineal glands were harvested at the indicated time of day and whole pineal protein extracts were analyzed by anti-c-$Fos_{129-153}$ immunoblotting. Animals were exposed to darkness (L → D) or to light (L → L) at night. Animals that had been SCGX'ed 2 weeks earlier were housed in a 14:10 light/dark lighting cycle (L → D). One group of SCGX animals was injected with isoproterenol (ISO, 1 mg/kg) 4 hours before death. In this and the following figure the migration of pre-stained protein molecular weight standards (Amersham, Arlington Heights, IL) is indicated on the right of each blot (kDa). [Reprinted with permission from Baler, R., and Klein, D.C., *J. Biol. Chem.* **270,** 27319–27325, 1995.]

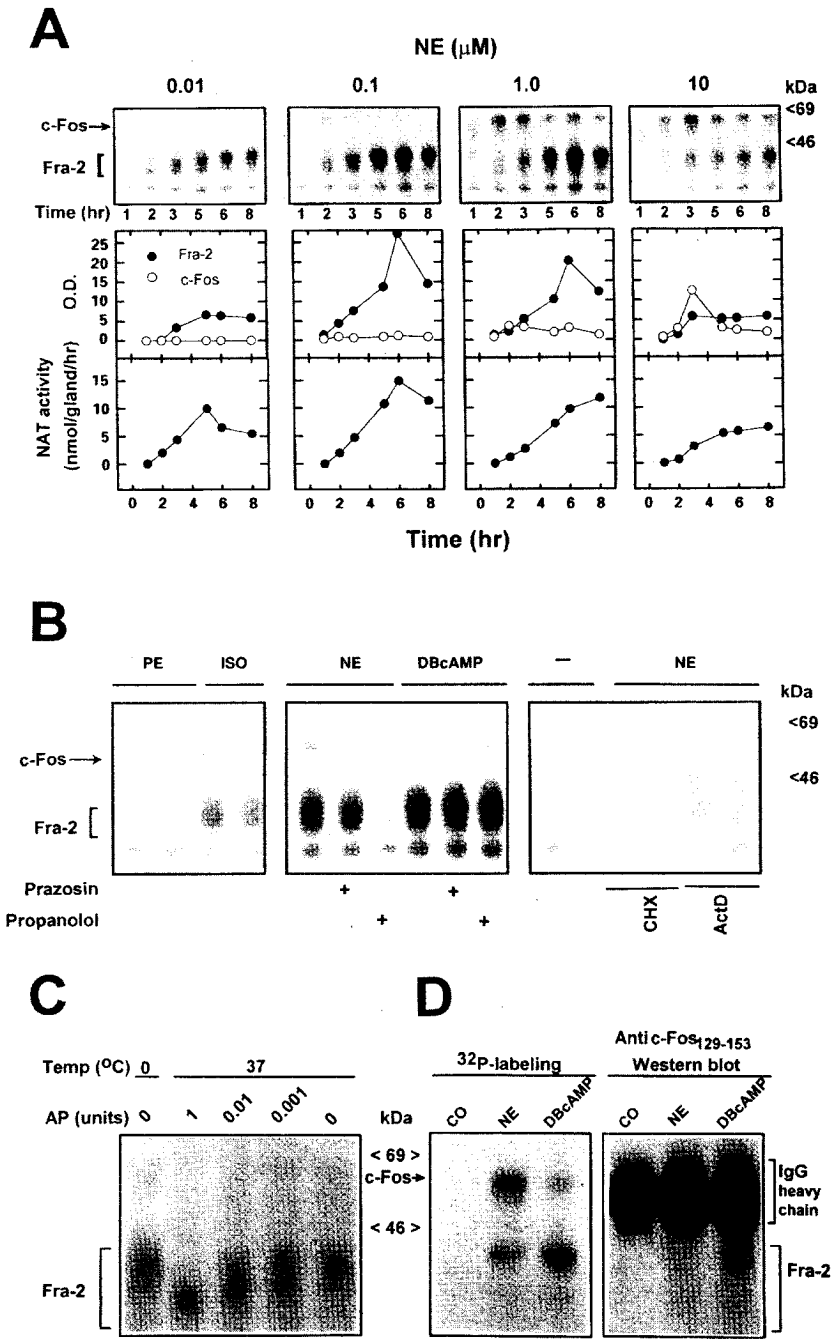

A
NE (μM)
0.01
0.1
1.0
10
kDa
<69
<46
c-Fos
Fra-2
Time (hr)
O.D.
Fra-2
c-Fos
NAT activity
(nmol/gland/hr)
Time (hr)
B
PE
ISO
NE
DBcAMP
—
NE
kDa
<69
<46
c-Fos
Fra-2
Prazosin
Propanolol
CHX
ActD
C
Temp (°C) 0
37
AP (units) 0
1
0.01
0.001
0
kDa
< 69 >
c-Fos
< 46 >
Fra-2
D
32P-labeling
Anti c-Fos129-153
Western blot
CO
NE
DBcAMP
IgG
heavy
chain
Fra-2

FIG. 24. Rat pineal Fra-2 is induced by a β-AR → cyclic AMP mechanism. (A) NE time course and dose response. Samples were analyzed by Western blot using anti-c-$Fos_{129-153}$ serum and processed as in Figure 23. Upper panel presents anti-c-$Fos_{129-153}$ immunoblotting of Fra-2 and c-Fos in protein extracts of glands treated with NE as indicated. The lower panels represent a densitometric quantitation of the Western blot data and NAT activity. (B) Effects of adrenergic agonists, protagonists, and antagonists on Fra-2 induction. Anti-c-$Fos_{129-153}$ immunoblotting analysis of whole-protein extracts from pineal glands (two glands per lane) that were treated with PE (0.1 μM), ISO (0.1 μM), NE (0.1 μM), or DBcAMP (1 mM) for 6 hours. Treatment with 5 μM prazosin, 5 μM propranolol, cycloheximide (CHX, 60 μg/ml) or Actinomycin D (ActD, 50 μg/ml) was started 30 minutes before stimulation. (C) Phosphatase treatment affects the migration of Fra-2. Five μl of a pineal nuclear extract prepared from NE-stimulated glands (0.1 μM, 6 hours), treated with the indicated amount of calf intestinal alkaline phosphatase (AP) at 37°C for 20 minutes or left untreated on ice and subjected to anti-c-$Fos_{129-153}$ immunoblotting. (D) Fra-2 is a phosphoprotein. Pineal glands in culture were stimulated with either NE (10 μM, 3 hours) or DBcAMP (1 mM, 6 hours) during incubation with ^{32}P NaO_3PO_4 (2 mCi/ml). Extracts were immunoprecipitated with anti-c-$Fos_{129-153}$, Fra's were identified by anti-c-$Fos_{129-153}$ immunoblotting (right panel) and autoradiography (left panel). [Reprinted with permission from Baler, R., and Klein, D.C., *J. Biol. Chem.* **270,** 27319–27325, 1995.]

of ICER protein in regulation of AA-NAT gene expression. This position is supported by the finding that the rhythmic pattern of pineal AA-NAT mRNA persists in mice who do not express ICER because the CREM gene has been knocked out (Foulkes *et al.,* 1996a; Sassone-Corsi, chapter 5, this volume).

Although ICER is not apparently responsible for rhythmicity in the expression of AA-NAT under a typical lighting cycle, it is likely that ICER determines the magnitude of the nocturnal increase in AA-NAT mRNA because it co-exists and competes with CREB for occupancy of CRE sites. This interpretation is supported by the finding that the amplitude of the AA-NAT mRNA rhythm is greater in CREM knockout mice (Foulkes *et al.,* 1996a; Sassone-Corsi, chapter 5, this volume). Although ICER may not play a role in rhythmicity under typical lighting conditions, ICER protein does exhibit 24-hour changes under extreme light/dark (L:D) cycles (4:20 and 20:4)(Foulkes *et al.,* 1996b) and it is possible that this represents an exaggeration of a subtle mechanism that functions under more natural lighting cycles to modulate seasonal changes in the AA-NAT response.

Accordingly it appears that expression of AA-NAT in the mouse and rat is turned on at the start of the night period by phosphorylation of CREB; that the maximal level of expression is determined by the relative abundance of ICER and CREB at the natCRE; and that expression is turned off actively by the rapid increase in Fra-2 protein and passively by dephosphorylation of CREB, which would occur towards the end of the night period.

D. CHICKEN

The chicken pineal gland is of special importance because it represents an opportunity for circadian biologists to learn about the molecular basis of clock

function. This is because the rhythm in melatonin production in the chicken is driven in part by a clock located within the pineal gland (Binkley *et al.*, 1978; Deguchi, 1979a; Takahashi *et al.*, 1980). We and other investigators in the field suspect that the clock → melatonin link may involve rhythmic expression of the AA-NAT gene. If this were the case it might be possible to get closer to clock molecules by using the AA-NAT promoter as a stepping stone. A better understanding of the chicken pineal circadian clock could lead to a better understanding of the SCN circadian clock. As discussed below in detail, this is also true for the pike and zebrafish pineal gland.

Chicken mRNA has been studied using a chicken-specific AA-NAT cDNA probe (Bernard *et al.*, 1997a). This has revealed that the transcript is easily detectable during the day in the pineal gland of the intact animal and that there is an ~ 10-fold increase in this mRNA during the night, which parallels the increase in enzyme activity (Fig. 25A). The rhythm in AA-NAT mRNA persists in both constant darkness and constant light (Fig. 25B and C). The persistence of the rhythm in constant light in the chicken is in contrast to what occurs in the rat (Fig. 13B), in that light completely blocks the nocturnal increase in rat pineal AA-NAT mRNA (Roseboom *et al.*, 1996). This presumably reflects light blockade of SCN stimulation of the pineal gland (Klein, 1985). It is interesting to note that, whereas the rat and chicken differ as regards the persistence of the rhythm in constant lighting, they are similar as regards the response to unexpected light at night in a normal lighting cycle. In both species light causes AA-NAT activity to decrease rapidly but does not cause a detectable change in AA-NAT mRNA levels (Klein and Weller, 1972; Binkley *et al.*, 1975; Figs. 14 and 26).

Our *in vivo* studies on the chicken pineal gland have also revealed that the peak in AA-NAT mRNA is delayed by ~ 6 hours after switching to constant light (Fig. 25C) and is advanced by ~ 6 hours in constant darkness when chickens are exposed to a pulse of light at the end of the night (Fig. 27). These phase-shifts appear to reflect the action of light on the circadian clock. Such results make it clear that the chicken pineal AA-NAT mRNA rhythm is driven by an endogenous clock mechanism. However, as indicated in the Introduction, there are multiple clocks in the chicken. In addition to the one in the pineal gland, other clocks are present in the avian homolog of the SCN (Cassone and Menaker, 1983; Cassone *et al.*, 1990) and in the retina (Zawilska and Iuvone, 1992; Thomas *et al.*, 1993; Iuvone, 1996).

To determine if the pineal clock regulates the rhythm in AA-NAT mRNA without the involvement of other structures, studies were done in cell culture (Bernard *et al.*, 1997b). Under these conditions a night/day rhythm in AA-NAT mRNA is generated, which can be entrained to environmental lighting cues (Fig. 28A). In addition the rhythm persists in constant darkness (Fig. 28B), indicating that the pineal clock actually regulates the rhythm in AA-NAT mRNA.

Analysis of the second messengers involved in regulating chicken melatonin production has indicated that cyclic AMP has a stimulatory influence. Elevation

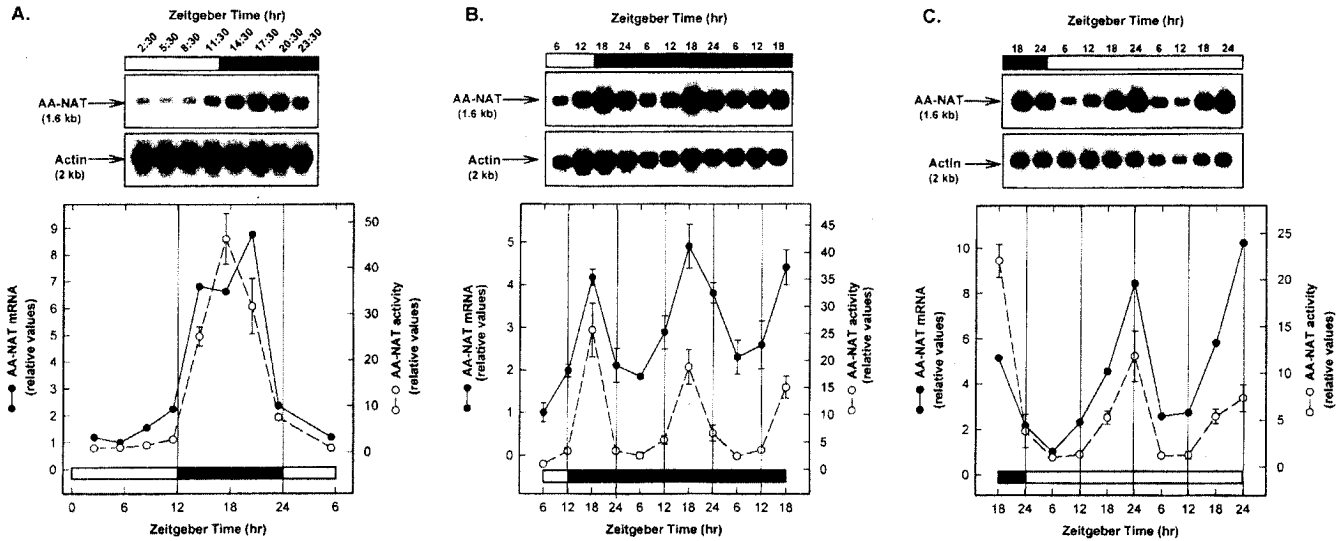


FIG. 25. Rhythm in pineal AA-NAT mRNA in the intact chicken. Animals used in these studies were housed for 2 weeks in controlled lighting (LD 12:12; lights on at ZT 0). Pineal glands were taken at the indicated times and processed for RNA analysis. The top panels show representative Northern blot analyses of AA-NAT and actin mRNAs (10 μg total RNA per lane). The bottom panels represent the quantitative analysis of the Northern blots (●-●) and the levels of AA-NAT activity (○-○) in each experimental group. The abundance of the AA-NAT transcript has been normalized to actin mRNA. (A) The abundance of chicken pineal AA-NAT mRNA exhibits a 24-hour rhythm in LD. The filled bar indicates when lights were off and the vertical lines indicate the light/dark transitions. All values represent the mean of duplicate determinations (RNA) or the mean ± SEM (activity; n = 6) and are expressed relative to the ZT 5:30 timepoint values. The ZT 5:30 values of AA-NAT mRNA and activity have been plotted twice. The levels of AA-NAT activity were 80 ± 10 pmoles/min/mg protein. (B) Rhythm in pineal AA-NAT mRNA persists in constant darkness (DD). Following 2 weeks in LD 12:12, chickens were transferred to DD. The filled bar indicates when lights were off and the vertical lines indicate the subjective day/night transitions. The zeitgeber times of the subjective cycle are indicated as a reference. All values represent the mean ± SEM of three (RNA) or seven (activity) determinations and are expressed relative to the first ZT 6 (light) timepoint value. The levels of AA-NAT activity at ZT6 were 115 ± 22 pmoles/min/mg protein. (C) Rhythm in pineal AA-NAT mRNA persists in constant light (LL). Following 2 weeks in LD 12:12, chickens were transferred to LL. The open bar indicates when lights were on and the vertical lines indicate the subjective day/night transitions. All values represent the mean of duplicate determinations (RNA) or the mean ± SEM (activity; n = 4 pineal glands per group) and are expressed relative to the value measured 6 hours after the onset of light (first ZT 6 with light). The levels of AA-NAT activity at the reference timepoint were 180 ± 30 pmoles/min/mg protein. [Reprinted with permission from Bernard, M., Iuvone, P.M., Cassone, V.M., Roseboom, P.H., Coon, S.L., and Klein, D.C. *J. Neurochem.* **68,** 213–224, 1997.]

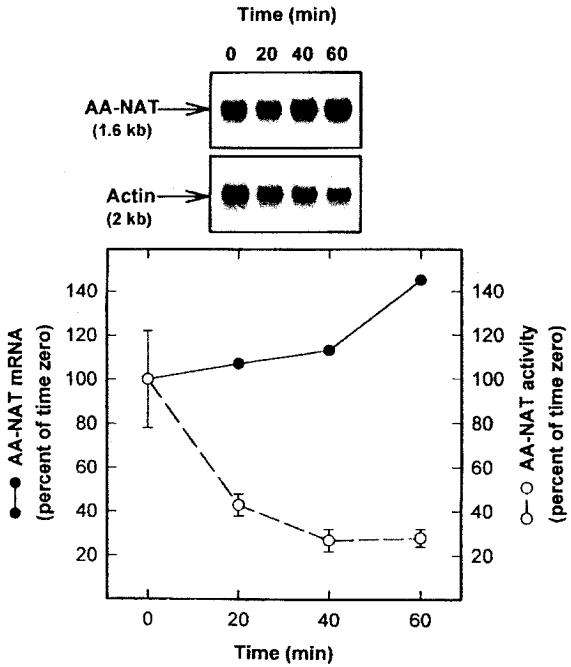


FIG. 26. Acute effect of light at night on levels of chicken AA-NAT mRNA and activity. Chickens were housed in a controlled lighting environment (LD 12:12; lights on at ZT 0). Starting at ZT 18 (= midnight) the animals were exposed to fluorescent light for the indicated times. Pineal glands were taken and processed for RNA analysis. The top panel shows representative Northern blot analysis of AA-NAT and actin mRNAs (10 μg total RNA/lane). The bottom panel represents the quantitative analysis of the Northern blot (●-●) and the levels of AA-NAT activity (o-o) in each experimental group. The abundance of the AA-NAT transcript has been normalized to actin mRNA. Values represent the mean of duplicate determinations (RNA) or the mean ± SEM (activity; n = 4 pineal glands per group) and are expressed as percent of the t = 0 timepoint (ZT 18). The levels of AA-NAT activity at ZT 18 were 2910 ± 130 pmoles/min/mg protein. [Reprinted with permission from Bernard, M., Iuvone, P.M., Cassone, V.M., Roseboom, P.H., Coon, S.L., and Klein, D.C. *J. Neurochem.* **68,** 213–224, 1997.]

of cyclic AMP due to forskolin treatment strongly increases AA-NAT activity (Deguchi, 1979b; Zatz and Mullen, 1988; Takahashi *et al.,* 1989). However, *in vitro* studies have revealed that cyclic AMP has relatively little influence on AA-NAT mRNA (Bernard *et al.,* 1997b). Although a small positive response is evident, forskolin treatment does not markedly enhance the nocturnal increase in AA-NAT mRNA nor does it increase the abundance of AA-NAT mRNA during the day (Fig. 29). This suggests to us that cyclic AMP influences chick AA-NAT

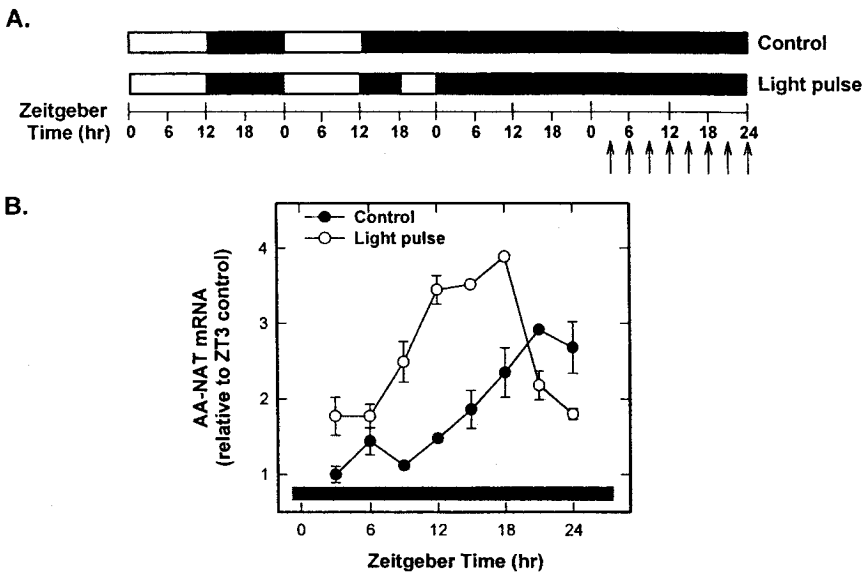


FIG. 27. Phase-shifting effect of light on the chicken pineal AA-NAT mRNA rhythm. (A) Schematic representation of the experiment. Chickens were housed in a controlled lighting environment (LD 12:12; lights on at ZT 0) and were then transferred to DD ("Control"). Some chickens were exposed to light for 6 hours in the late "night" after being transferred to DD ("Light pulse"). The open bars indicate when lights were on. Zeitgeber time of the subjective cycle is indicated. Pineal glands were obtained at the times indicated by the arrows. (B) Quantitative analysis of a representative Northern blot is presented. The abundance of the AA-NAT mRNA transcript has been normalized to actin mRNA. All values represent the mean ± SEM of three determinations and are expressed relative to the ZT 3 timepoint value (= first timepoint; control chickens). [Reprinted with permission from Bernard, M., Iuvone, P.M., Cassone, V.M., Roseboom, P.H., Coon, S.L., and Klein, D.C. *J. Neurochem.* **68,** 213–224, 1997.]

activity primarily through post-transcriptional mechanisms and, therefore, that cyclic AMP does not drive the rhythm in AA-NAT mRNA.

These *in vitro* studies have also revealed that treatments with protein synthesis inhibitors cause a marked increase in AA-NAT mRNA levels, while it completely abolishes the nocturnal or forskolin-induced increase in enzyme activity, as expected (Fig. 30). This stimulatory effect of protein synthesis inhibitors on AA-NAT mRNA is evident throughout the circadian cycle (Fig. 31). Accordingly it appears that a rapidly turning over protein is present throughout the day that suppresses AA-NAT mRNA. This protein could be an RNase, an inhibitory transcription factor, or another negatively acting protein component of the regulatory machinery. The role of such a protein in the rhythmic changes in AA-NAT mRNA is not clear.

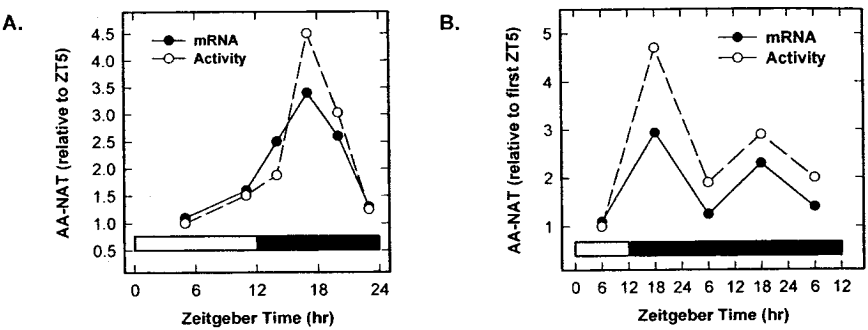


FIG. 28. Rhythm in AA-NAT mRNA and activity in cultured chick pinealocytes. (A) Dissociated pineal cells from day-old chicks were cultured for 5 days in a white light/red light cycle (L:R 12:12, white light on at ZT 0) and were harvested at the indicated times. Red light is used for practical purposes; chick pinealocytes exhibits similar circadian rhythms in both constant darkness and constant red light. The filled bar indicates when white light was off. For each timepoint, total RNA was prepared from two groups of $\sim 6 \times 10^6$ cells and analyzed by Northern blot. Quantitative analysis of representative Northern blot is presented (●-●). AA-NAT activity was measured at each time point (○-○). All values (mRNA and activity) represent the mean of duplicate determinations and are expressed relative to the ZT5 timepoint. (B) Dissociated pineal cells from day-old chicks were cultured for 4 days in LR 12:12 and were transferred to constant red (RR). The filled bar indicates when white light was off. Cells were harvested at the indicated times (zeitgeber times are indicated as a reference) and were processed for Northern blot. Quantitative analysis of a Northern blot is presented (●-●). In each experimental group AA-NAT values have been normalized to the G3PDH signal. AA-NAT activity was measured at each timepoint (○-○). All values (mRNA and activity) represent the mean of duplicate determinations and are expressed relative to the first ZT5 timepoint. [Reprinted with permission from Bernard, M., Klein, D.C., and Zatz, M., *Proc. Natl. Acad. Sci. U.S.A.* **94,** 304–309, 1997. Copyright 1997 National Academy of Sciences, U.S.A.]

Future work in the chicken pineal gland should help identify the transcription factors responsible for rhythmic expression of the AA-NAT gene; ultimately this will bring us closer to a molecular understanding of vertebrate clock function.

E. FISH

Fish represent an interesting group of vertebrates as regards melatonin production because, in some species such as the trout, the rhythmic changes in melatonin production occur in direct response to light (Gern and Greenhouse, 1988) and, in others such as pike and zebrafish, a pineal clock drives these changes (Falcon *et al.,* 1992; Cahill, 1996). In addition the melatonin-synthesizing cells are photoreceptors that share many biochemical and anatomical features found in the retina (Falcón *et al.*, 1992).

1. Trout

Analysis of the trout pineal mRNA using a trout cDNA probe indicates that AA-NAT mRNA exists as multiple forms; the relative abundance of these forms

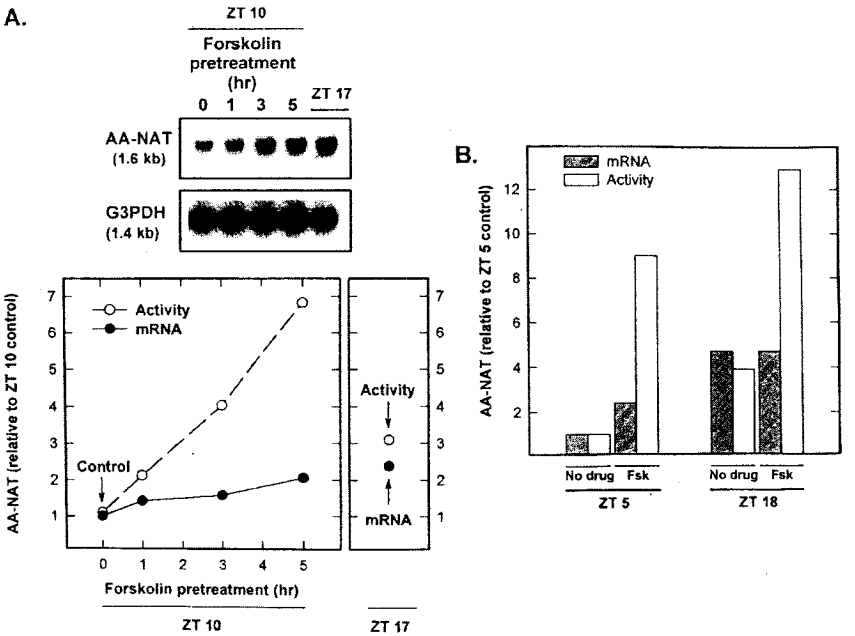


FIG. 29. Effect of forskolin stimulation on the levels of AA-NAT mRNA and activity in the chick pinealocyte. Dissociated pineal cells from day-old chicks were cultured for 4 days in LR 12:12 (white light on at ZT 0). (A) On the fourth day groups of cells were incubated for the indicated times with 1 μM forskolin (Fsk). All treatments were ended at ZT 10. In each experimental group total RNA was prepared from two groups of ~ 6 × 10^6 cells. The top panel illustrates a representative Northern blot analysis of AA-NAT and G3PDH mRNAs (20 μg total RNA per lane). The bottom panel shows the quantitative analysis of the Northern blot (●-●). In each experimental group AA-NAT values have been normalized to the G3PDH signal. AA-NAT activity was measured at each time point (○-○). The symbols (●) and (○) on the right panel represent, respectively, the levels of AA-NAT mRNA and activity in untreated cells harvested at ZT 17. All values (mRNA and activity) represent the mean of duplicate determinations and are expressed relative to the ZT10 control time point (= end of treatment). (B) On the fourth day of culture cells were incubated for 6 hours with 1 μM forskolin either during the day (ZT23-ZT5) or during the night (ZT12-ZT18). Analysis of AA-NAT mRNA and activity were as described in (A). All values represent the mean ± SEM of three separate experiments and are expressed relative to the ZT 5 control time point. [Reprinted with permission from Bernard, M., Klein, D.C., and Zatz, M., *Proc. Natl. Acad. Sci. U.S.A.* **94**, 304–309, 1997. Copyright 1997 National Academy of Sciences, U.S.A.]

is 2.4 kb > 2.8 kb ≈ 2.1 kb >> 1.6 kb. Similar Northern blot analysis of the retina failed to detect a transcript. *In vivo* and *in vitro* studies indicate there is no day/night difference in the abundance of pineal AA-NAT mRNA (Figs. 32 and 33). The absence of changes in mRNA indicates that day/night changes seen in enzyme activity are due to posttranscriptional mechanisms and that a photo-

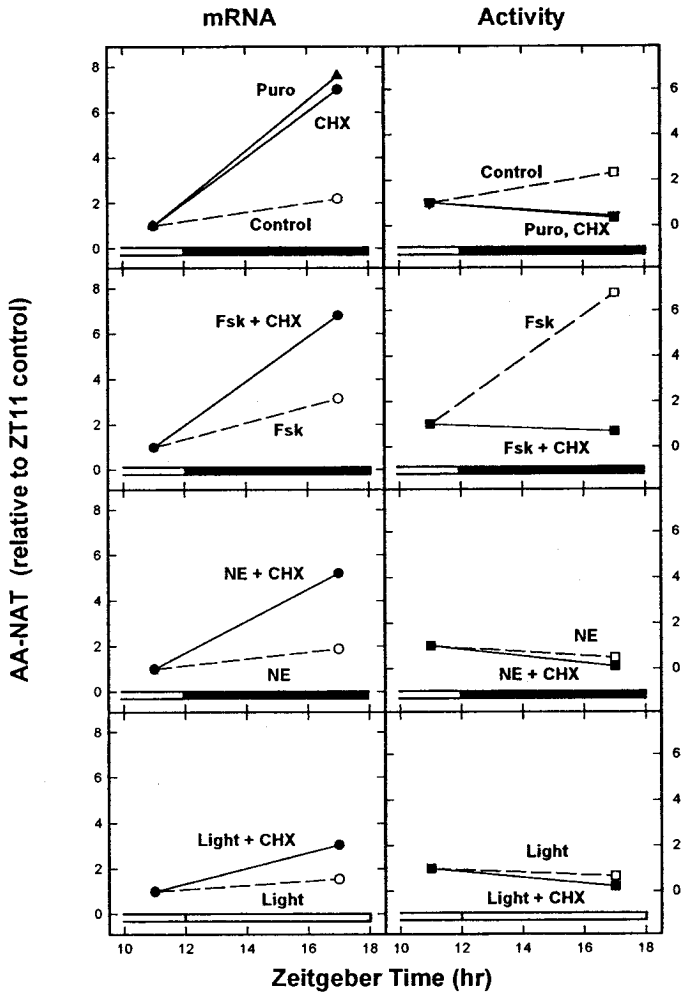


FIG. 30. Effect of co-treatments of chick pinealocytes with protein synthesis inhibitors and forskolin, norepinephrine, or light on AA-NAT mRNA and activity. Dissociated pineal cells from day-old chicks were cultured for 5 days in LR and then were incubated for 6 hours with 1 μM forskolin (Fsk) or 10 μM norepinephrine (NE), in the presence or absence of 10 μM cycloheximide (CHX) (or 50 μg/ml puromycin). All treatments were performed at the beginning of the night (ZT11-ZT17). For one group of cells the CHX treatment was performed in the presence of light. Total RNA was prepared from two groups of ~ 6×10^6 cells and analyzed by Northern blot (20 μg total RNA per lane). AA-NAT mRNA values have been normalized to the G3PDH signal (left panel). AA-NAT activity was also measured in each group (right panel). All values (mRNA and activity) represent the mean of duplicate determinations and are expressed relative to the ZT 11 control. [Reprinted with permission from Bernard, M., Klein, D.C., and Zatz, M., *Proc. Natl. Acad. Sci. U.S.A.* **94,** 304–309, 1997. Copyright 1997 National Academy of Sciences, U.S.A.]

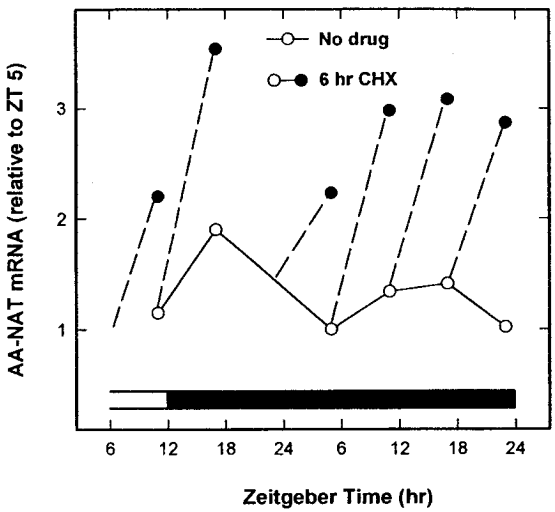


FIG. 31. Effect of protein synthesis inhibitors on chick pinealocyte AA-NAT mRNA levels. Dissociated pineal cells from day-old chicks were cultured for 5 days in LR 12:12 and were then transferred to constant red. At different times of the subjective cycle (zeitgeber times are indicated as a reference) cells were incubated with 10 μM cycloheximide (CHX) for 6 hours. Total RNA was prepared from two groups of ~ 6 × 10^6 cells and analyzed by Northern blot (20 μg total RNA per lane). AA-NAT mRNA values have been normalized to the G3PDH signal. All values represent the mean of duplicate determinations and are expressed relative to the ZT 5 control (after transfer to RR). [Reprinted with permission from Bernard, M., Klein, D.C., and Zatz, M., *Proc. Natl. Acad. Sci. U.S.A.* **94,** 304–309, 1997. Copyright 1997 National Academy of Sciences, U.S.A.]

receptor → second messenger → AA-NAT gene pathway may not exist in the trout pineal.

2. *Pike*

Although the pike is closely related to the trout phylogenetically, regulation of melatonin synthesis in these species is quite different. Melatonin synthesis in the pike is regulated by a clock mechanism located within the photosensitive melatonin-synthesizing pinealocytes; accordingly, the pike pinealocyte is a self-contained photosensitive circadian system with both a clock and an output (Bolliet *et al.,* 1997), as is true of the chicken pinealocyte. Pike pineal AA-NAT mRNA is 2.1 and 2.8 kb (Fig. 32). The abundance of pike pineal AA-NAT mRNA is higher at night than during the day. This pattern is maintained in constant light, indicating that the rhythm is driven by an internal clock. *In vivo* and *in vitro* studies indicate that AA-NAT activity displays a rhythm of similar amplitude under typical day/night lighting cycles or under constant darkness, whereas as a

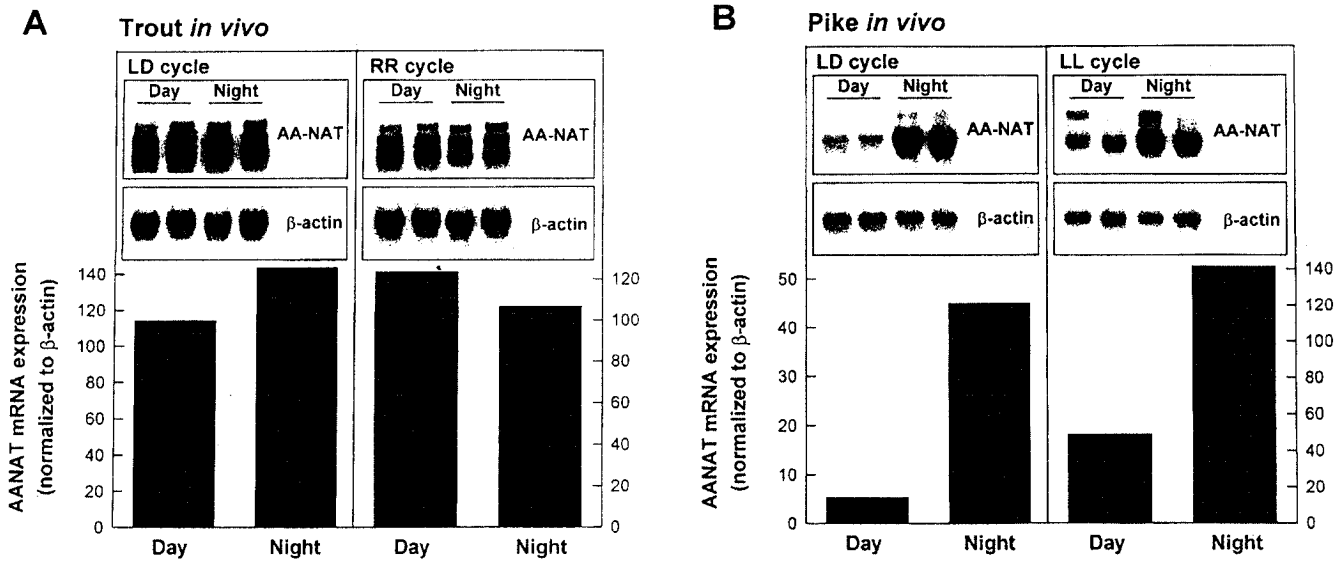


FIG. 32. Regulation of pineal AA-NAT mRNA in intact fish. Trout (A) and pike (B) were maintained in aquaria under a controlled white light/red light cycle (LR 11:13, white light on at 0800). Some trout were maintained under constant red lighting (RR) and some pike were housed under constant white lighting (LL). Trout pineal glands were collected at 1200 (day) and 2400 (night) and pike glands were collected at 1200 (day) and 0400 (night). Pineal glands were analyzed for AA-NAT mRNA expression. Each lane contains either 15 μg of total RNA from a pool of five trout pineal glands or 10 μg from a pool of two to three pike pineal glands. The blots were probed with a ^{32}P-labeled, random-primed fragment of trout AA-NAT (clone T3), then stripped and probed for β-actin. Northern blot images are shown in the insets and AA-NAT expression normalized to actin is shown in the histogram. [From V. Bégay, S.L. Coon, J. Falcón, and D.C. Klein, unpublished results.]

very low amplitude rhythm persists under constant light (Falcón *et al.*, 1992). Accordingly, it appears that the pike pineal clock generates a rhythm in AA-NAT mRNA under all lighting conditions and that this is translated into a rhythm in AA-NAT activity in a typical lighting cycle and in constant darkness, but not under conditions of constant lighting.

3. *Zebrafish*

The zebrafish has important potential for genetic studies. Investigators in the field hope to monitor clock activity by following *in situ* emission of light from light-generating constructs whose expression is clock regulated. This approach is attractive in the zebrafish because it may be possible to monitor rhythmic gene expression in the pineal gland in intact animals using simple photodetection devices because the fish is transparent.

Melatonin synthesis in the zebrafish exhibits a daily rhythm that is under the control of a pineal clock (Cahill, 1996). Using a trout cDNA probe it has been determined that AA-NAT mRNA exists as a single transcript (1.7 kb). Analysis of the abundance of mRNA encoding AA-NAT indicates it exhibits a daily rhythm *in vitro* and is controlled by the pineal clock because the rhythm persists in constant darkness (Fig. 33).

VIII. Summary

An overview of the progress made in understanding AA-NAT in a number of vertebrate species reveals several conserved features and some remarkable differences. Conservation is evident in the fundamental structure of the protein, especially in the core element, which is presumed to be involved in substrate binding and catalysis. The remarkably high conservation of one region, the putative arylalkylamine binding domain, probably explains the high substrate specificity of AA-NAT. Conservation of the two putative PKA sites probably reflects conserved cyclic AMP regulatory mechanisms and is consistent with cyclic AMP regulation being a conserved feature of AA-NAT regulation. Another element that appears to be conserved is a close correlation between enzyme activity and enzyme protein and that rapid changes in activity are accompanied by rapid changes in protein.

In contrast to the high degree of conservation of some features of the biology of AA-NAT, it is evident that highly diverse regulatory mechanisms exist. In mammals the rhythm is driven by a clock in the SCN. Neural stimulation at night results in an increase in release of NE, which elevates cyclic AMP; in sheep, cyclic AMP may act primarily through post-transcriptional mechanisms, whereas in the rat, transcriptional mechanisms are also involved. In the chicken, a pineal

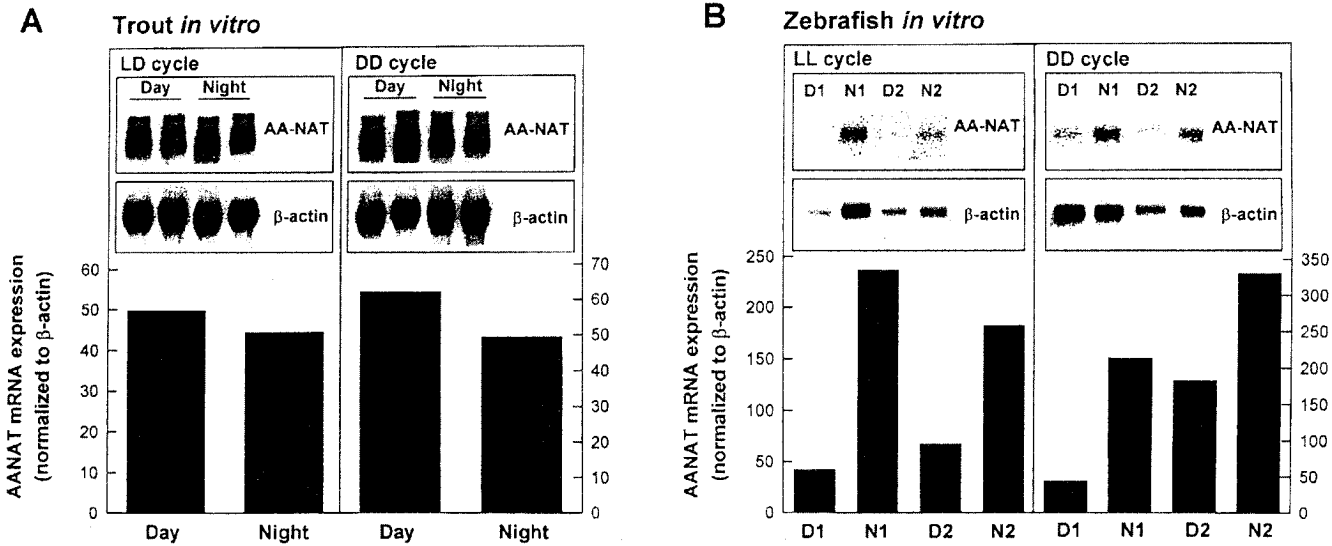


FIG. 33. Regulation of AA-NAT mRNA in the cultured fish pineal gland. (A) Trout pineal glands were removed during the light period and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 15°C under 5% CO_2. Glands were maintained for 3 days under either a light/dark cycle (LD 11:13, light on at 0800) or constant darkness (DD). Day and Night samples were collected at 1200 and 2400, respectively. (B) Zebrafish pineal glands were removed during the light period and cultured as decribed (Cahill, 1996). Glands were maintained for 3 days under controlled light/dark cycle (LD 12:12, light on at 0600) or constant darkness (DD). Samples were collected at 1200 and 2400 during the second and third subjective days (D1, D2) and nights (N1, N2). Trout and zebrafish pineal glands were analyzed for AA-NAT mRNA levels. For the trout pineal sample each lane contains 15 μg of total RNA from a pool of four to five glands; for the zebrafish pineal sample each lane contains 1.5 μg of total RNA from a pool of 40–50 glands. The blots were probed with a ^{32}P-labeled, random-primed fragment of trout AA-NAT (clone T3), then stripped and probed for β-actin. Northern blot images are shown in the insets and AA-NAT expression normalized to actin is shown in the histogram. [From V. Bégay, G. Cahill, J. Falcón, S.L. Coon, and D.C. Klein, unpublished results.]

clock participates in the regulation of AA-NAT mRNA and activity and seems to be responsible for increasing activity at night; however, other clocks are involved and, in this species, the release of NE occurs during the day and causes a decrease in cyclic AMP. In the chicken, as in the rat, cyclic AMP has a post-transcriptional influence; however, in contrast to the rat, cyclic AMP has only a very weak influence on transcription. Differences exist among fish as well. In trout, it appears that AA-NAT mRNA is expressed at a high level without a rhythmic component and that changes in activity occur without the involvement of a clock, apparently at a post-transcriptional level. One possibility is that light acts directly on pineal photoreceptors to decrease cyclic AMP—which, in turn, regulates the amount of AA-NAT protein and activity—and that this is the primary regulatory mechanism. The pike and zebrafish pineal glands appear to be different because clocks exist within these structures to regulate both AA-NAT mRNA and activity.

The cloning of cDNA encoding AA-NAT has resulted in important advances and in significant progress in understanding the regulation of this enzyme. It has also made it quite clear that the nocturnal increase in melatonin is of essential importance for vertebrate physiology because the integrity of this signal has been preserved as a constant feature throughout vertebrate evolution and diverse mechanisms have developed in different species to produce this signal.

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DISCUSSION

William Chin: If you blind a chicken, do the melatonin rhythms persist?

David Klein: The melatonin rhythm persists in blind chickens because the clocks that regulate the pineal keep on ticking—the clock in the brain and the clock in the pineal gland itself. However, these clocks have rhythms that are not exactly 24 hours. The periods are 24 ± 0.5 hours; this phenomenon is called a "free-running" rhythm.

Paolo Sassone-Corsi: FRA-2 is a member of the fos family and, as you know, it cannot homodimerize. What is the FRA-2 partner in this case? If there is one, does it oscillate?

David Klein: The partner is Jun-B (R. Baler and D.C. Klein, in preparation). David Carter has shown Jun-B mRNA oscillates but little is known about the protein.

Paolo Sassone-Corsi: Where does the FRA-2 complex bind and what does it do to block cAMP-induced transcription?

David Klein: We think it binds at AP-1 sites on the promoter close to the CRE site and has the overall effect of blocking the polymerase.

Michael Thorner: Please tell us whether NAT changes in pituitary and whether it has a circadian rhythm.

David Klein: We have not yet analyzed sheep pituitary glands on a day/night basis. But we're now are in the process of collecting samples, so stay tuned!

Roger Dilts: Explain the differences you might expect between nocturnal animals such as rats and humans where activity decreases in dark. And the relevance of using nocturnal animals in relationship to humans.

David Klein: Remarkably, there is no relationship between the melatonin rhythm and lifestyle or locomotor activity. The rhythm in melatonin is only linked to environmental lighting and, for that reason, melatonin is called the "hormone of the night."